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PHYSICAL AND CHEMICAL CONSTANTS OF THE EGG OF THE SEA URCHIN, ARBACIA PUNCTULATA

E. NEWTON HARVEY

PRINCETON UNIVERSITY

For purposes of calculation it would frequently be convenient to have readily accessible data regarding the physical characteristics of much-used animal and plant cells. The unfertilized egg of the sea urchin, *Arbacia punctulata*, is a cell in a relatively stable condition, which has been studied perhaps more widely than any other marine material. Accordingly, I have collected the available data regarding this egg, with the hope that others will do a similar thing for different cells and organisms. A table of physiological constants for man should prove most valuable.

The figures for the unfertilized sea urchin egg are given in metric units for the temperature indicated (mostly "room" temperature, about 23° C.) with temperature coefficients where these are known. Although the cm.-gram-second units are common in physics, the micron-milligram-minute units give more convenient figures in dealing with single cells and have been more widely used by physiologists. It must always be remembered that there is considerable variation in the eggs of different females and less variation in the eggs of a single female (see Goldforb, 1917; for effects of ageing, Goldforb, 1918, *a* and *b*). The most probable average value is, therefore, taken as a basis for derived values. Some determinations cannot be made with great accuracy and consequently represent approximations, such as the diameter of granules and percentage of granules, and determinations based on such measurements. Where our knowledge of data is not complete, this compilation may serve as a guide to future investigation.

1. DIMENSIONS

Egg diameter,[1] 74 μ, average of 550 cells from four females. Eggs do not flatten under their own weight (McCutcheon, Lucké and Hart-

[1] The European *Arbacia pustulosa* egg has a diameter of about 100 μ.

line, 1931); 66 to 94 μ (Glaser, 1914). Loss of diameter on fertilization, 2.3 μ for 74 μ egg (Glaser, 1924).[2]

Egg surface, 17200 μ^2.

Egg volume, 212000 μ^3.

Diameter and volume of eggs separated by centrifugal force. Colorless and pigmented half separate from whole egg and clear and granular quarter from colorless half (E. B. Harvey, 1932*a*).

	Diameter μ	Volume μ^3	
Whole egg	73	203690	(197400 μ^3 Lucké, 1932)
Colorless half egg	60	113100	(108600 μ^3 Lucké, 1932)
Pigmented half egg	56	91950	(90700 μ^3 Lucké, 1932)
Clear quarter egg	53	77950	
Granular quarter egg	40	33510	

Thickness of jelly, 28 to 32 μ (E. B. Harvey).[3]

Thickness of perivitelline space, 3–5 μ (E. B. Harvey).

Thickness of hyaline plasma layer (ectoplasmic layer), 1–2 μ (E. B. Harvey).

Diameter nucleus, 11.5 μ (E. B. Harvey).

Diameter oil globules, 1 μ (E. B. Harvey).

Diameter fifth layer granules,[4] 0.6–1 μ (E. B. Harvey).

Diameter yolk granules,[5] 0.7–1.1 μ (E. B. Harvey); 0.3 μ (Heilbrunn, 1926).

Diameter pigment granules, 1.1–1.6 μ (E. B. Harvey); 0.92 μ (Heilbrunn, 1926).

Percentage formed bodies (E. N. Harvey).[6] Nucleus 0.4; oil globules 1.0; fifth layer 4.8; yolk 27.2; pigment 5.5; fluid 61.1.

Number of chromosomes (diploid), probably 38 (Morgan, 1927).

2. Densities

Sea water, 1.024 (Lyon, 1907). 1.02426 21.5° C./21.5° C. (Garrey, 1905).

[2] This has been questioned by others.

[3] The undated references to E. B. Harvey apply to data furnished by her for this article but not published elsewhere.

[4] This layer appears on top of the yolk layer when eggs are centrifuged for a long time. The granules are very variable in size and shape. The figures for granule diameter are only approximate.

[5] These granules are often irregular and frequently polyhedral in centrifuged eggs.

[6] The percentage nucleus was calculated from its volume based on diameter measurements. The percentage of other formed bodies was made by measuring the dimensions of the spherical segment occupied by the layers in long centrifuged eggs, calculating the volume of the spherical segment and taking 74 per cent of this, since spheres of equal size lying in a volume occupy 74 per cent of that volume. The figures must be considered only approximate, as it is not certain how much packing of the granules occurs.

Egg with jelly, 1.090 (E. N. Harvey, 1931).[7] **Egg without jelly,** 1.084 (E. N. Harvey, 1931).[7] } 1.081–1.087 (Lyon, 1907).[8] 1.0485–1.0656 (Heilbrunn, 1926*b*).[9]

Density oil? [10]

Density nucleus?

Density clear layer, 1.0358–1.0514 (Heilbrunn, 1926*b*).[11]

Density fifth layer granules?

Density yolk granules, 1.1035–1.1269 (Heilbrunn, 1926*b*).[12]

Density pigment granules?

Density clear half of egg separated by centrifugal force, 1.076 (E. N. Harvey, 1931).[7]

Density yolk half of egg separated by centrifugal force, > 1.100 (E. N. Harvey, 1931).[7]

3. Viscosity

Water = 0.01 poise (dyne-seconds per cm.^{2}) at 20° C.

Granule-free protoplasm, 0.018–0.025 (approximately .02) poise [13] (Heilbrunn, 1926, *a* and *b*). Granule-free protoplasm, 0.04 (Heilbrunn, 1928).[14] (See this book for effect of substances on viscosity.)

Entire protoplasm, 2–3 times above.

Fertilized egg considerably more viscous. For changes during development see Heilbrunn, 1920.

For effect of CO_2 see Jacobs, 1922; for effect of fatty acid-salt buffers see Howard, 1931; for effect of acids and alkalies see Barth, 1929; for effect of temperature see Heilbrunn, 1924.

4. Tension at Surface

Air/water surface tension = 73 dynes per cm. at 20° C.

Unfertilized egg less than 0.2 dynes per cm., with considerable

[7] Determined by centrifuging eggs in mixtures of sea water and .95*m* cane sugar at 22° C. and measuring the density of the sea water-sugar mixture in which they remain suspended with a hydrometer graduated for 60° F./60° F. There is considerable variation in density of different eggs. *m* = molal, 342 grams cane sugar to a liter of water.

[8] Determined by centrifuging eggs in gum arabic in sea water. No difference in density up to 16-cell stage but pluteus has a density of 1.055 to 1.066.

[9] Determined by centrifuging eggs in cane sugar solutions whose densities were determined from tables.

[10] The relative density of oil, nucleus, etc., is in the order given, oil and nucleus being lighter than clear layer, the remaining granules heavier.

[11] Calculated from density of whole egg and volume (19 per cent) occupied by heavy granules of egg.

[12] Determined by crushing eggs and centrifuging crushed material in sugar solutions of various densities.

[13] Determined by centrifuging granules and applying Stokes' law with Cunningham correction.

[14] Determined by Brownian movement method.

variation, for 25 per cent increase in surface area.[15] (E. N. Harvey, 1931.)

Initial tension 0.08 dynes per cm. for undistorted egg (fertilized or unfertilized) and approximately proportional to deformation (flattening) and to increase in surface area.[16] Surface has properties of elastic membrane. Increase of surface force for 1 per cent increase of surface area is 0.005 dynes per cm. for unfertilized and 0.7 dynes per cm. for fertilized egg. Internal pressure about 40 dynes per cm.[2] (Cole, 1932.)

5. Osmotic Properties

Osmotic pressure equals that of sea water (22 atmospheres at 0° C.), whose depression of freezing point, Δ, = − 1.81° C. (− 1.805 to − 1.84). NaCl 0.52M, $MgCl_2$ 0.29M and cane sugar 0.73M (342.2 grams per liter of solution) have same Δ as sea water. (Garrey, 1905, 1915.)

Equilibrium given by $(V_0 - b)P_0 = (V_{ex} - b)P_{ex}$, where V_0 = volume in sea water, P_0 = osmotic pressure sea water, V_{ex} = volume in concentrated or diluted sea water, P_{ex} = osmotic pressure in concentrated or diluted sea water, b = osmotically inactive material, 11 per cent (7 to 14 per cent) (McCutcheon, Lucké and Hartline, 1931). No effect of narcotics on equilibrium.

Permeability (k) **to water** defined as:

$$\frac{dV}{dt} = kA(P - P_{ex}),$$

where dV/dt = rate of change in volume, A = surface area, P = osmotic pressure at time t, P_{ex} = osmotic pressure within egg or of solution with which cell is in equilibrium. For water entering eggs at 20° C., $k = 0.087\ \mu^3$ per μ^2 surface per atmosphere difference of pressure per minute; for water leaving eggs, $k = 0.141\ \mu^3$. At 15° C., k = 0.05–0.06 for endosmosis and 0.07–0.08 for exosmosis. Values are independent of osmotic pressure but depend on kind and proportions of salt in medium, injury, narcotics, etc. Q_{10} = 2 to 3 between 12° and 24° C. Thermal μ = 15200. (Lucké, Hartline and McCutcheon, 1931.) See also Northrop, 1927; McCutcheon and Lucké, 1926 and 1927.

For fertilized eggs k = 0.12 to 0.17 at 15° C. for endosmosis (Lucké, unpublished).

Lillie (1916; 1917) finds water enters fertilized eggs and butyric acid activated eggs four times more rapidly than unfertilized.

[15] Determined by centrifugal force necessary to pull egg into two halves.
[16] Determined by force necessary to deform eggs by flattening.

Absence of ions (glucose solution) increases k from 0.05 to 0.1 at 12° C. and 0.0001M $CaCl_2$ or $MgCl_2$ added to glucose solution maintains k same as in sea water (McCutcheon and Lucké, 1928).

Cations decrease permeability to water, the effectiveness increasing with the valence of the cation. In 0.38*m* dextrose solution containing 0.005M K_3 citrate (in which solution cells have high water permeability), the following concentrations of cobaltammine chlorides were required to reduce permeability to the value obtained in sea water:— 0.00005M of the 6 valent salt, more than twice as much of the 4 valent salt, more than eight times as much of the 3 valent, and 64 times as much of the 2 valent salt, while this amount of the 1 valent salt was incompletely effective. Temperature 12° ± 0.5° C. (Lucké and McCutcheon, 1929).

Anions increase permeability to water, the effectiveness increasing rapidly with the valence of the anions. In 0.38*m* dextrose solution containing 0.0005M $CaCl_2$, 0.001M of potassium ferrocyanide was required to definitely increase permeability, twice as much ferricyanide, four times as much potassium sulphate, and eight times as much chloride. Temperature 12° ± 0.5° C. (Lucké and McCutcheon, 1929).

Narcotics (urethanes and carbamates) in sea water do not decrease permeability to water beyond the value normally found in sea water. When dissolved in a non-electrolyte solution, they tend to decrease permeability to water. Thus, 0.025M *n*-butyl carbamate in 0.38*m* dextrose solution decreased permeability from 0.096 (the value in dextrose solution alone) to 0.062. Temperature 15° ± 0.5° C. (Lucké, 1931).

For effects of HCN and KCN on permeability to water see Blumenthal, 1927; for ether see Heilbrunn, 1925; for anaesthetics and KCN see also Lillie, R. S., 1918, and Blumenthal, 1928.

6. Permeability

Permeability to a solute (S) may be defined as

$$\frac{dS}{dt} = kA\left(C_e - \frac{S}{V}\right)$$

where dS/dt = rate of change of amount of solute; A = surface area; C_e = external concentration; V = volume of the egg. k = number of mols that will penetrate $1\mu^2$ of surface in 1 minute with a concentration difference between exterior and interior of 1 mol per liter and is [17] (Jacobs and Stewart, 1932):

[17] Determined by change in volume of eggs in hypertonic solutions.

Ethylene glycol	3.6×10^{-15}
Acetamid	5.8×10^{-15}
Propionamid	14.2×10^{-15}
Butyramid	36.6×10^{-15}
Glycerol	0.5×10^{-15}

For fertilized eggs k for ethylene glycol $= 9.8 \times 10^{-15}$ (Stewart and Jacobs, 1932).

For permeability to non-electrolytes and NH_4 salts see Stewart, 1931; for fatty acid-salt buffers see Howard, 1931.

7. Electrical Properties

Electrical resistance.[18]—Interior 90 ohm-cm. or 3.6 times that of sea water at room temperature for 1000 to 15×10^6 cycles. Impedance of surface high below 1000 cycles and behaves like a "polarization-capacity." No measurable change in resistance on fertilization (Cole, 1928).

Dielectric constant?

Cataphoretic potential,[19] $-$ 0.035 volts with jelly; $-$ 0.021 volts without jelly for zeta potential (K. Dan, 1931).

8. Hydrogen-ion Concentration

pH of sea water, 8.2.

pH of egg? Probably 6.6–6.8 from studies of other echinoderms by indicator method. No change on fertilization. Change to 5.4–5.6 on injury. Nucleus of immature egg 7.6–7.8 (Chambers and Pollak, 1927; Needham, 1926).

Buffer value?

9. Metabolism

Heat production,[20] $-$ 0.08 gram-cal. per 10^6 eggs of 74 μ diameter per hour; 0.88 gram-cal. on fertilization, falling to 0.58 gram-cal. 20 minutes after fertilization and 0.52 gram-cal. per 10^6 eggs per hour at first cleavage (50 minutes after fertilization). 3.34 ergs per egg per hour for unfertilized and 20 ergs per egg per hour for fertilized eggs (Rogers and Cole, 1925).

Carbon-dioxide production? See Lyon, 1904.

Oxygen consumption, 33.6 mm.^{3} (17–51 mm.^{3}) measured at 24.7° C. O_2 per 10^6 eggs (diameter 72–80 μ; average 77 μ) per hour at 24.7° C. or 0.0023 mm.^{3} O_2 per mm.^{3} egg per minute. Fertilized eggs four times with no variation during cleavage [21] (Tang, 1931). 30 mm. per 10^6 eggs (diameter 72 μ) per hour at 25° C. .0025 mm.^{3} O_2 per mm.^{3}

[18] Determined by vacuum thermocouple voltmeter-ammeter method.
[19] Determined by deflection from vertical fall in electric field.
[20] Determined by thermocouple and twin-calorimeters.
[21] Warburg manometer method.

egg per minute. Fertilized eggs 5 times greater with no variation during cleavage [21] (Tang and Gerard).

Temperature coefficient of oxygen consumption [22] (Loeb and Wasteneys, 1911*a*).

° C.	Q_{10}	° C.	Q_{10}	° C.	Q_{10}
3–13.....	2.18	10–20.....	2.17	17–27.....	2.0
5–15.....	2.16	13–23.....	2.45	20–30.....	1.96
7–17.....	2.00	15–25.....	2.24		

Oxygen consumption of unfertilized eggs at different O_2 tensions. 760 to 50 mm. O_2, 100 per cent; 45 mm., 95 per cent; 20 mm., 90 per cent; 10 mm., 70 per cent; 6 mm., 55 per cent; 2 mm., 40 per cent (Tang, 1931) [21] at 24.7° C.; see Gerard, 1931.

Oxygen consumption of fertilized eggs at different O_2 tensions. 228 to 80 mm. O_2, 100 per cent; 20 mm., 90 per cent; 13 mm., 80 per cent; 9 mm., 60 per cent; 4 mm., 40 per cent; 2 mm., 20 per cent. Below 11 mm. division rate slows; below 4 mm. division rate ceases [23] (Amberson, 1928). Temperature about 22°. 760 to 50 mm., 100 per cent; 28 mm., 80 per cent; 20 mm., 60 per cent; 15 mm., 40 per cent; 9 mm., 20 per cent (Tang and Gerard).[21] Temperature 25°.

For effect of methylene blue alone, with cyanide and with narcotics, see Barron, 1929.

For effect of CO_2 and HCl on oxygen consumption, see Root, 1930.

For oxygen consumption under various conditions see McClendon and Mitchell, 1912; Loeb and Wasteneys, 1911*b*, 1913, 1915; Wasteneys, 1916.

10. Oxidation-Reduction Potential

Not determined for *Arbacia* but from other sea urchin eggs. rH = < 7.9 in nitrogen and 12 ($E_0' = -0.06$ volts for pH = 7) (Chambers, Pollak and Cohen, 1929, 1931) or 21–22 (Needham, 1926) in air by indicator method. No change on fertilization and during segmentation.

Red pigment, echinochrome. $E_0 = +.1995$ volts at pH = 0, decreasing 0.06 volts for each pH unit except above pH = 8.78 where slope changes due to acid-base dissociation (Cannan, 1927).

11. Composition

Total nitrogen?

Total fat, carbohydrate and protein?

[22] Winkler method.

[23] Haldane analysis of gas in equilibrium with sea water.

Lactic acid, 3.14 mg. per gram egg protein ($N_2 \times 6.25$). 81 per cent increase if treated with 0.003M KCN for 3 hours.[24] 19 per cent more lactic acid in 4 to 8-cell fertilized eggs (Perlzweig and Barron, 1928).

Free sugar absent (Perlzweig and Barron, 1928).

Reducing sugar by acid hydrolysis = 50 mg. glucose per gram egg protein (Perlzweig and Barron, 1928).

Total solid, 18.1 per cent (McClendon, 1909).

Ash, 8.5–10 per cent dry weight of eggs (Page, 1927).

Calcium,[25] 1.9 mg. per 10^6 eggs; 0.047 millimols; 10^6 eggs dried weigh 124 mg. (Page, 1927).

Magnesium,[26] 4.48 mg. per 10^6 eggs; 0.182 millimols (Page, 1927).

Sodium,[27] 1.301 mg. per 10^6 eggs; 0.056 millimols (Page, 1927).

Potassium,[28] 2.445 mg. per 10^6 eggs; 0.063 millimols (Page, 1927).

Iron,[29] 0.030 mg. per 10^6 eggs; 0.0005 millimols (Page, 1927).

Sulphate,[30] 0.00046 mg. per 10^6 eggs; 0.00004 millimols (Page, 1927).

Chloride,[31] 0.1864 mg. per 10^6 eggs; 0.0053 millimols (Page, 1927).

Total phosphate,[32] 0.9064 mg. per 10^6 eggs; 0.0291 millimols (Page, 1927).

Acid-soluble P, 0.40–0.50 mg. per 10^6 eggs; 0.0161 millimols (Page, unpublished).

Lipoid P, 0.498 mg. per 10^6 eggs; 0.160 millimols (Page, unpublished).

Nitrate, trace (Page, 1927).

Silica, moderate amounts (Page, 1927).

Copper, 17 micrograms per cc. eggs for unripe ovarian eggs; 175 micrograms per cc. for unfertilized and 21 micrograms per cc. for fertilized eggs (Glaser, 1923).

Cholesterol, present (Mathews, 1913).

Oil; iodine number 146–148; saponification value 606 (Page, 1927).

Echinochrome; see McClendon, 1912.

Catalase content of unfertilized and fertilized eggs same (Amberg and Winternitz, 1911); see Lyon, 1909.

Composition of layers of crushed and centrifuged unfertilized eggs (with jelly) in percentages of weight of whole egg mass (P = phosphorus, N = nitrogen) (McClendon, 1909).

[24] Nitrogen determined by micro-Kjeldahl and lactic acid by Clausen's method.
[25] Precipitated by NH_4 oxalate and ignited to CaO.
[26] Weighed as $Mg_2P_2O_7$.
[27] Kramer and Gittlemann pyroantimonate method.
[28] Kramer and Tisdall method.
[29] Allen-Scott colorimetric method.
[30] Weighed as $BaSO_4$.
[31] Mohr method.
[32] Benedict-Theis method.

Layer	Centripetal 32.5	Centrifugal 67.5 [33]	Whole Egg
Water	28.6	53.3	81.9
Solids	3.9	14.2	18.1
Ether ext.	0.308	1.946	2.254
P in ether ext.	0.00154	0.06760	0.06914
Alcohol ext.	1.60	3.48	5.08
P in alcohol ext.	0.0434	0.0814	0.1248
Water ext.	0.78	1.42	2.20
P in water ext.	0.130	0.182	0.312
Residue of water ext.	1.309	7.29	8.59
P in residue	0.0392	0.1167	0.1559
N in residue	0.1625	0.7750	0.9375
Ash in residue	0.0162	0.0264	0.0426
Total *P* in layers	0.21414	0.4478	0.66194

[33] Contains jelly.

Analysis of eggs washed with isotonic glucose (K. C. Blanchard, unpublished).

Ash . . . . . 7–8 per cent dry weight of eggs
Calcium . . . . . 6.45 ± 0.3 per cent of ash
Magnesium . . . . . 1.77 ± 0.02 per cent of ash
Potassium . . . . . 15.79 ± 0.25 per cent of ash
Lactic acid . . . . . 0.4 mg. (when eggs first shed) to 6 mg. (on standing) per gram egg protein
Glycogen . . . . . 50–80 mg. per gram egg protein

For analysis of autolysed eggs see Lyon and Shackell, 1910.

For egg secretions see Lillie, F. R., 1913; Glaser, 1914, 1921, 1922; Woodward, 1918; Clowes and Bachman, 1921.

12. Rate of Development

Time in minutes from fertilization to first cleavage (Loeb and Wasteneys, 1911*a*).

°C.	Time	°C.	Time	°C.	Time
7	498	16	85	26	33
8	410	17.5	70	27.5	34
9	308	18	68	30	33
10	217	20	56	31	37
12	147	22	47	32	No cleavage
15	100	25	40	35	No cleavage

°C.	Q_{10}	°C.	Q_{10}	°C.	Q_{10}
7–17	7.3	10–20	3.9	15–25	2.6
8–18	6	12–22	3.3	16.26	2.6
9–19	> 4	13–23	3.3	17.5–27.5	2.2
				20–30	1.7

Time from fertilization to 50 per cent first cleavage, 62.5 minutes at 20.6–20.8° C. (Haywood and Root, 1930); 55 minutes to 50 per cent first cleavage and 83 minutes to second cleavage at 22° C. (Lillie and Cattell, 1923); 50 minutes to 50 per cent first cleavage at 22° C. (Whitaker, 1929).

Time from fertilization at 23° C. to various stages of development (E. B. Harvey).

Completion of fertilization membrane, 1 to 2 minutes.
Hyaline plasma layer begins 2 minutes.
Union of pronuclei (monaster), 10 minutes.
Nuclear streak begins 20, ends 40, marked 25–30 minutes.
Nuclear membrane disappears, 40 minutes.
Amphiaster, 45 minutes.
First cleavage (50 per cent), 50 minutes.
Second cleavage (50 per cent), 78 minutes.
Third cleavage (50 per cent), 103 minutes.

For effect of sea water diluted with .73M cane sugar see Lillie and Cattell, 1923.

For effect of CO_2 and acids on development see Clowes and Smith, 1923, Smith and Clowes, 1924, *a* and *b*, and Smith, 1925.

For effect of CO_2 on cleavage rate see Haywood, 1927, and Haywood and Root, 1930.

For effect of lack of oxygen see Harvey, E. B., 1930, and Lyon, 1902.

For effect of $HgCl_2$ see Hoadley, 1930.

For effect of mechanical shocks see Whitney, 1906.

For cleavage rates of centrifuged egg fragments see Harvey, E. B., 1932*a*, and Whitaker, 1929.

13. Narcosis

Anaesthetic and Lethal Concentrations (Heilbrunn, 1920) of

	Anaesthetic *per cent*	Lethal *per cent*
Ether	2	4
Chloroform	0.13	1
Chloral hydrate	0.25	1
Nitromethane	2	3
Paraldehyde	4	8
Acetone	5	10
Ethyl nitrate	0.4	?
Ethyl acetate	2	5
Ethyl butyrate	0.25	0.5
Acetonitrile	4	5
N Propyl alcohol	1	?
Amyl alcohol	0.6	1
Phenyl urethane	4/5 sat.	sat.
Ethyl urethane	1.5	3

Critical narcotic concentrations for reversibly suppressing cleavage (Harvey, E. B., 1932*b*).

Ethyl urethane	0.15–0.2 M
N-propyl urethane	0.07 M
Isopropyl urethane	0.1 M
N-butyl carbamate	0.025 M
Isoamyl carbamate	0.01 M
Phenyl urethane	0.00125–0.0025 M

Critical narcotic concentrations for reversibly suppressing cleavage (Lillie, 1914).

Methyl urethane	2–2.5 per cent; .29–.33 M
Ethyl urethane	1.5–1.75 per cent; .15–.19 M
Phenyl urethane	.08–.06 per cent; .005–.006 M
Ethyl alcohol	5 vol. per cent; .87 M
Propyl alcohol	2 vol. per cent; .27 M
Isopropyl alcohol	3 vol. per cent; .4 M
H-Butyl alcohol	.8 vol. per cent; .086 M
Isoamyl alcohol	.4 vol. per cent; .037 M
Capryl alcohol	.015 vol. per cent; .001 M
Ethyl ether	.5–.6 vol. per cent; .05–.06 M
Chloroform	.06 per cent; 1/12 saturated; .005 M
Chloral hydrate	.1–.12 per cent; .006–.007 M
Chloretone	.2–.25 per cent; .008–.01 M

See also Baldwin, 1920, for effect of alcohols on dividing eggs.

14. Miscellaneous

For **effects of light** see Lillie and Baskervill, 1921, 1922, and Hinrichs, 1926, 1927.

For **effect of salts** see Lillie, R. S., 1910, 1911; Lillie and Baskervill, 1921.

For **effect of supersound waves** see Harvey, Harvey and Loomis, 1928, and Harvey and Loomis, 1931.

For **rhythms of susceptibility during development** see Lyon, 1904, Baldwin, 1920, and Page, 1929; Lillie, R. S., 1916 and Just, 1928, for hypotonic sea water; Moore, A. R., 1915, for hypertonic sea water.

For **initiation of development** and related phenomena see Greeley, 1902; Hunter, 1903; Harvey, E. N., 1910; Loeb, 1913, 1915; Heilbrunn, 1913, 1915, 1920, *a* and *b*, 1925; Lillie, F. R., 1914, 1921; Glaser, 1914; Moore, C. R., 1916, 1917; Just, 1922, 1928, *a* and *b*, 1929.

For **surface precipitation reaction** see Heilbrunn, 1930.

For **agglutination** see Goldforb, 1929.

For **nature of fertilization membrane** see Kite, 1912; Heilbrunn, 1913, 1915, 1924*a*; Harvey, E. N., 1914; McClendon, 1914; Garrey, 1919; Chambers, 1921.

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THE DEVELOPMENT OF HALF AND QUARTER EGGS OF ARBACIA PUNCTULATA AND OF STRONGLY CENTRIFUGED WHOLE EGGS

ETHEL BROWNE HARVEY

(From the Marine Biological Laboratory, Woods Hole, and Washington Square College, New York University)

When the unfertilized eggs of *Arbacia punctulata* are strongly centrifuged in a sugar solution of the same (or graded) density, they become dumb-bell shaped and then separate into two nearly equal spheres or half-eggs, one colorless and the other pigmented (E. N. Harvey, 1931). Further, when the colorless spheres are again centrifuged, more strongly, in a sugar solution of the same (or graded) density, they separate into two spheres or quarter-eggs, one without visible granules and the other with granules. All of these half- and quarter-eggs, as well as the deformed total eggs just before pulling apart, can be fertilized and develop. We can in this way obtain "egg fragments" of very definite size and content in great numbers. This obviates the former tedious method of cutting individual eggs with a glass needle (Harnley, 1926; Hörstadius, 1928; Plough, 1929; Tennent, Taylor and Whitaker, 1929; Whitaker, 1929) and insures a much more accurate division of the egg into parts of known structure than the old haphazard and harmful method of shaking the eggs into pieces (Hertwigs, Boveri, Driesch, Morgan, etc.).[1] Sea urchin eggs centrifuged in sea water alone do not separate into spheres owing to the fact that they sink to the bottom and are crushed by the force exerted on them. The method employed was to put two parts 0.95 molal sugar (95 per cent of 342 grams cane sugar added to 1 liter tap water, an isotonic solution) in the centrifuge tubes and above this one part of sea water containing eggs; the tubes were rolled gently to obtain partial mixing and then centrifuged at about 7000 r.p.m. (11 cm. radius) for four minutes. On removal of the tubes, the colorless spheres formed a whitish layer just beneath the surface, the whole eggs, which had not broken apart, formed a reddish layer about halfway down the tube and the pigmented spheres rested at the bottom of the tubes. One could then pipette off any one of the three kinds of eggs with practically no admixture of the other varieties. The sugar solution was found to have no ill effect on the eggs, since eggs could be kept in this solution for at least five hours

[1] The centrifuge method of separating eggs supplements rather than supplants the cutting method, since the granular constitution of fragments is different in the two cases.

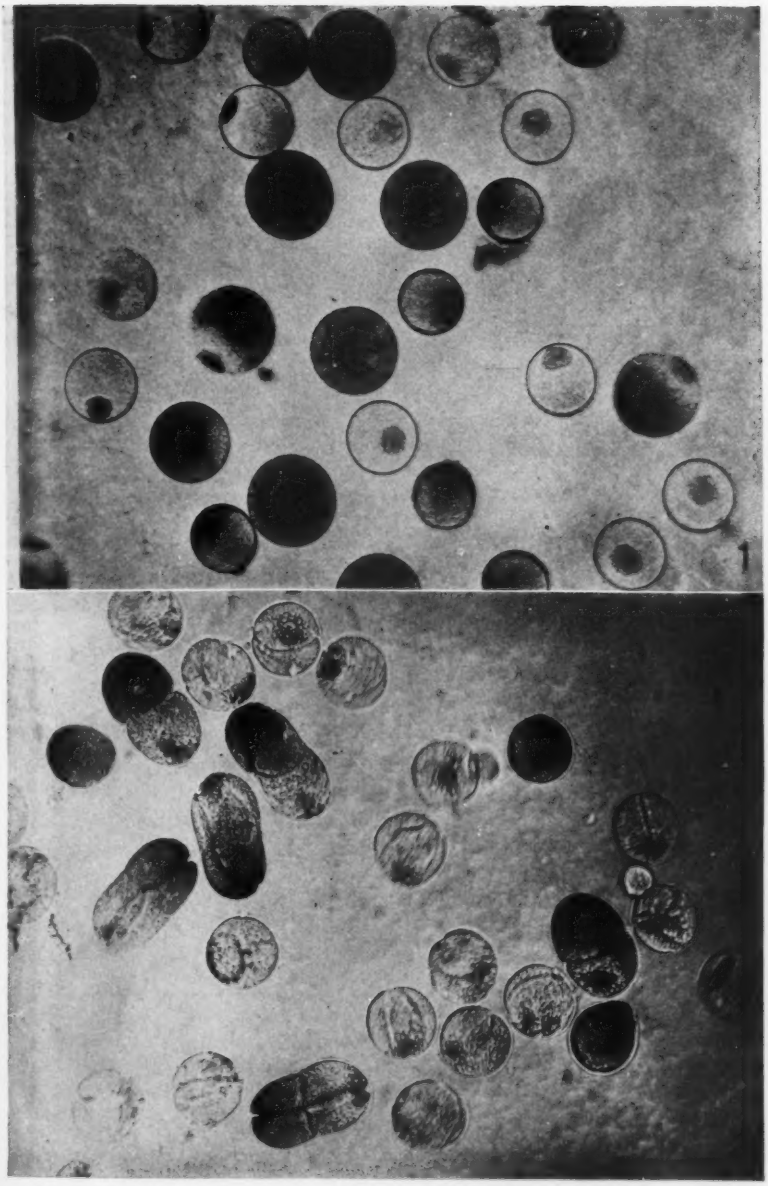
and when returned to sea water could be fertilized, and developed normally. Eggs cannot be fertilized, however, while in the sugar solution although the sperms are active and surround the egg. The rapid centrifuging also has no ill effect upon the eggs, as eggs subjected to this treatment develop into normal plutei when fertilized in sea water. To separate the colorless half-eggs into two parts, they were taken from the centrifuge tubes and put into other centrifuge tubes above a small amount of cane sugar solution (3 parts of 0.95 molal sugar to 1 part sea water) and a little sea water placed above. The tubes were then centrifuged for 45 minutes at about 10,000 r.p.m. (radius 11 cm.)

Unfertilized Whole, Half- and Quarter-eggs

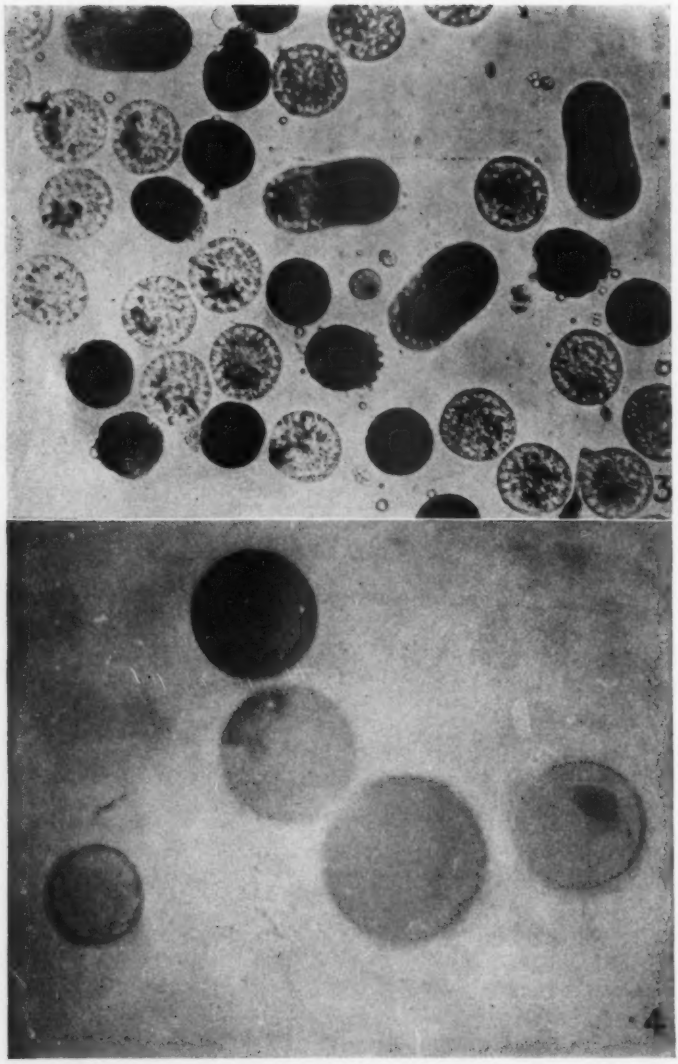
When normal *Arbacia* eggs are centrifuged as described above, they become stratified while still spherical into five layers, (1) oil on top, (2) a clear layer without visible (*i.e.*, under ordinary illumination) granules, in which lies the nucleus, (3) a thin granular layer separated out only with strong centrifugal force, and not heretofore described and which we have, therefore, termed "the fifth layer," (4) a large yellowish yolk layer, and (5) a layer of red pigment concentrated at the heavier pole (Fig. 25). The fifth layer can be very beautifully demonstrated by staining with methyl violet or methyl green when it becomes purple, or with Janus green when it becomes green.

The eggs after stratification become elongate, then dumb-bell shaped (Fig. 25) and then break into two slightly unequal spheres, usually through the upper part of the yolk. The colorless sphere (cf. Fig. 1), slightly larger, contains therefore the oil, below which always lies the nucleus; the clear layer, without visible granules; the granular or fifth layer; and a little yolk at the heavier pole. The pigmented sphere, (cf. Fig. 13) slightly smaller, contains usually only yolk and pigment, the pigment being massed at the heavier pole; there is, of course, no nucleus in this sphere. In a few batches of eggs, the two spheres were almost equal in size, the separation having taken place in the fifth layer, a little of which appeared as a light cap on the pigmented sphere. Usually, however, there is quite an appreciable difference in size of the two half-eggs, and their relative size is fairly constant in any one centrifuged lot. When the colorless spheres are centrifuged again, as described above, they separate into (1) a larger sphere with oil, nucleus and clear layer—this quarter-egg now having practically no visible granules (Fig. 38); and (2) a quite small sphere composed entirely of the granules of the fifth layer and yolk, about half of each (cf. Fig. 46).

The average measurements obtained in typical lots of eggs are given in Table I. A photograph of unfertilized whole eggs and the two



Photograph 1. Unfertilized whole and half-eggs.
Photograph 2. 2 and 4-cell whole and colorless half-eggs.



Photograph 3. Blastulae of whole and colorless half-eggs.
Photograph 4. Five types of eggs, whole, half- and quarter-eggs.

kinds of half-eggs is given in Photograph 1. Photograph 4 is a picture of the five types of eggs, whole, half- and quarter-eggs.

Development of Half-eggs

Both the colorless and pigmented half-eggs can be fertilized in sea water immediately after centrifuging or at any later time. They both form fertilization membranes at the same time as the whole egg. These are often well separated from the surface of the egg but sometimes, especially in the pigmented spheres, rather closely investing. There is in both half-eggs a well marked ectoplasmic or hyaloplasmic layer formed on fertilization. The development of the two half-eggs must now be followed separately. All observations were made on living material, and the times given are for 23° C., when 50 per cent normal eggs cleave in 50 minutes.

The development of the colorless half-egg is quite normal. Many of the nuclear phenomena accompanying fertilization and cleavage can be seen with great clearness, and they parallel those described by

TABLE I

	Diameter	Volume	Sums of Volumes
	μ	μ³	μ³
Whole Egg	73	203700	
Colorless Half Egg	60	113100	205050
Pigmented Half Egg	56	91950	
Clear Quarter Egg	53	77950	111450
Granular Quarter Egg	40	33500	

Wilson (1895) in the living *Toxopneustes* egg, which is devoid of pigment and quite transparent; these phenomena cannot be observed in the normal living *Arbacia* egg on account of the pigment. The sperm aster can be seen in the granular area a few minutes after fertilization, and its approach toward the female pronucleus can be followed (Fig. 2). The female pronucleus travels down from its position under the oil cap toward the center of the egg about eight minutes after fertilization (Fig. 2), and as it moves down some of the oil spherules also move down from the oil cap. After the union of the pronuclei (Fig. 3) astral radiations extend through the granular area; in fact all the granules are often arranged in rays extending in a half circle from the nucleus (Fig. 4); there is no indication of rays (in the living egg) in the clear area free of granules. In the normal uncentrifuged egg at this period, the astral radiations extend throughout the cell, this being the "monaster stage" (Fig. 36). These radiations

gradually fade out and the nucleus enlarges from a diameter of 12 μ to 16 μ (Fig. 5). This is the "streak stage" of the normal *Arbacia* egg, lasting from 20 to 40 minutes after fertilization, characterized by a curved band extending on either side of the nucleus (Fig. 37); this stage is referred to by Wilson in the *Toxopneustes* egg as the "pause." The nuclear wall now breaks down (Fig. 6) and soon afterwards there are again radiations, now from the two poles of the amphiaster, but present only in the granular zone (Figs. 7, 8). The half-eggs divide about the same time as normal control eggs, or sometimes a little earlier as Whitaker (1929) found for his diploid fragments; the plane of cleavage usually comes in perpendicular to the stratification (Fig. 9), but sometimes parallel with it (Fig. 10) or in any intermediate position, but it divides the egg into two equal parts (photograph 2) as in the normal egg. The following cleavages come in at right angles to the preceding and divide the blastomeres equally (Figs. 11, 12). Blastulae (photograph 3) are formed from the half-eggs, quite normal except for size and coloring. In many of the cultures the larvae remained for several days as very actively swimming blastulae; in some they developed into gastrulae and in some into plutei with well developed skeleton and arms exactly like the normal ones except that they were colorless and only half the size.

The pigmented spheres have no nucleus at the time of fertilization. The aster accompanying the sperm nucleus may very often be seen in the yolk 15–20 minutes after the fertilization membrane has been given off (Fig. 13). The nucleus, at first very small, enlarges and is quite noticeable about 30 minutes after fertilization (Fig. 14), and about the time of first cleavage of the whole and colorless half-eggs. This single nucleus now usually without radiations, after enlarging considerably (Fig. 15), breaks down about one hour after fertilization (Fig. 16). At about 80 minutes after fertilization (Fig. 19), when the total and colorless spheres are in the 4-cell stage, many of the pigmented spheres have two nuclei; either a dumb-bell shaped nucleus or a very small amphiaster can sometimes be seen preceding the binucleate stage (Figs. 17, 18). Usually no cleavage plane comes in, probably owing to the mechanical difficulty in cutting through the dense material. After several successive divisions of the nuclei, the pigmented half-eggs appear as reddish spheres containing many white circles, some with radiations, giving somewhat the appearance of pictures of the moon with craters on the surface (Fig. 20). There are often slight indications of cleavage planes running in from the periphery as indentations or notches (Figs. 21, 22). Rather rarely, the division planes come in quite normally and the egg divides into 2, 4 and 8 equal blastomeres

(Figs. 23, 24). The first cleavage plane in these cases may come in in any relation to the stratification, and the following planes at right angles to the preceding. Although it is difficult to determine in living material, it would seem that when cleavage planes do not come in at first, they come in later on after some 20 or 30 nuclei are formed, for the blastulae seem quite normal and appear, as far as I could tell, multicellular. The blastulae are quite active, but are not very viable, and relatively few in any batch develop much further. I have, however, had a number of quite normal gastrulae and some plutei with skeletons, but only two with well developed arms. None have survived more than nine days. There seems no doubt that these merogonic eggs *can* give rise to dwarf embryos similar to normal ones except for containing more pigment. Whether the failure of the majority of these eggs to develop far is due to lack of certain formative stuffs or to the lack of the female pronucleus, or to the over-crowding with dense material is not certain, but I should judge from the appearance and behavior of the developing eggs that the last explanation is the correct one. It may be, however, that only those half-eggs containing some of the fifth layer develop, but this awaits further investigation.

It would seem then that both half-eggs can develop into plutei, and that neither food material (or very little) nor a female pronucleus is necessary for development.

Development of Strongly Centrifuged Whole Eggs

When the unfertilized *Arbacia* eggs are strongly centrifuged, as mentioned before, they elongate, then become dumb-bell shaped before breaking apart (Fig. 25). When these are left either in the sugar solution or in sea water, they soon become oval and then spherical, often within an hour. But if they are removed to sea water and fertilized immediately, they retain their dumb-bell shape and the fertilization membrane follows the contour of the surface with usually a bulge at one or both poles. This must be due to a "setting" or gelation of the protoplasm following fertilization, for if the fertilization membranes are removed by drawing the eggs into a capillary pipette, the eggs retain their aspherical shape (and develop). This is another indication of increased viscosity following fertilization. One can often observe the male aster in the yolk or granular zone, and this apparently pulls some pigment granules along as it travels toward the center (Figs. 26, 27). The descent of the female pronucleus can be observed and the pull of the oil spheres toward the center, the union of the two pronuclei and the gradual fading out of the sperm aster whose radiations have been visible only in the granular zone (Fig. 27), the enlarge-

ment of the nucleus and its rupture (Figs. 29, 30). A very striking stage is shown in Fig. 28, a sharp line of demarcation running between the clear zone and the granular zone; this corresponds to the "streak" stage in the normal egg (Fig. 37). A streak is only rarely seen running perpendicular to the stratification in well centrifuged eggs, probably owing to the denseness of the material. Later, rays from the amphiaster are seen in the yolk and granules of the fifth layer, which by this time are thoroughly mixed (Fig. 31); either a whole aster whose mate is sometimes faintly distinguishable in the clear zone into which some granules have spread, or less commonly two half asters in the granular and yolk zone. It would seem that there must be a change of axis in the mitotic figure, for in normal eggs the long axis of the spindle is the same as the long axis of the "streak," whereas here it is usually perpendicular to it. The first cleavage plane comes in at almost the same time as in normal eggs, usually through or near the constriction and parallel (or at a slight angle) with the stratification, separating one colorless blastomere from the one containing yolk and pigment (Fig. 32). These two cells are usually unequal in size, more frequently the colorless cell is the smaller, though the two blastomeres are sometimes of size corresponding with the two half-eggs, the cleavage plane following the future separation plane (Photograph 2). The first cleavage plane comes in rarely in these elongated eggs perpendicular to the stratification, and this is sometimes followed by a second cleavage plane also along the long axis resulting in four sausage shaped cells. The second cleavage plane always comes in perpendicular to the first (Fig. 33, Photograph 2). Many years ago, Lyon (1907) and Morgan and Lyon (1907) found that the first cleavage plane in centrifuged *Arbacia* eggs was usually perpendicular to the stratification. The apparent contradiction is explained by the difference in shape of the eggs. In *elongated* eggs, the first cleavage plane comes in usually parallel with the stratification, in the short axis. In *spherical* eggs it comes in perpendicular with the stratification; this is true both for eggs which are spherical because not centrifuged sufficiently to become elongate, and for eggs which have been elongate but have resumed a spherical shape on standing in sea water before fertilization. In slightly elongate or oval shaped eggs, the first cleavage plane comes in one way or the other in about equal numbers.

The later cleavage planes often come in fairly regularly except that the clear cells often divide in advance of the pigmented cells, and are smaller (Fig. 34). Very frequently, the original first cleavage plane remains quite prominent, the first two blastomeres developing almost independently (Fig. 35). So much so, in fact, that double embryos are

often produced, one colored and the other colorless, at first within the same membrane, and later swimming attached together. Slipper shaped blastulae (Photograph 3), normal gastrulae and plutei arise from the elongate and dumb-bell shaped eggs, but the stratification of pigment and yolk remains, and may be in any relation, apparently, to the axis of the embryo. Individual eggs have not been studied with reference to polarity.

Development of Quarter Eggs

The colorless half-eggs, when centrifuged again, are drawn out into dumb-bells (Fig. 51) and are then separated into spheres. The stratification is the same as before, the nucleus lying just below the oil cap in a large clear layer without visible granules; at the heavier pole is the granular or fifth layer and a layer of yolk. The half-egg breaks usually at the line between the clear layer and the granules, so that we obtain one perfectly clear sphere, with oil cap and nucleus (Fig. 38) and one granular sphere containing about an equal amount of granules (fifth layer) and yolk (cf. Fig. 46). This granular quarter-egg is much smaller than the clear quarter, about one sixth the volume of the whole egg.

Both of these quarter-eggs, as well as the dumb-bell shaped half-eggs can be fertilized in sea water, and throw off fertilization membranes. The ectoplasmic layer of the granular quarter is much thicker than that of the clear quarter, where it is thinner than in normal eggs.

Owing to the absence of granules in the clear quarter, nothing can usually be seen of the sperm aster; the female pronucleus can be observed migrating from the oil cap to the center of the egg about 25 minutes after fertilization, pulling some of the oil spheres along. This gradually enlarges from 12 μ to 16 μ, but no other change occurs for six hours or more (Fig. 39). In some eggs the nucleus becomes enormous, as large as 22 μ (nearly half the diameter of the egg, and an increase of six times in volume), but this is probably abnormal. The nucleus later breaks down and disappears (Fig. 40) and cleavage takes place some seven hours (or more) after fertilization (Figs. 41, 42) and in any plane with regard to the oil cap. The very slow cleavage of these diploid quarters is not in accord with Whitaker's (1929) explanation of cleavage rates. Other cleavages follow slowly, but usually the membrane breaks and there is a loose mass of cells, some perfectly clear and some with oil drops (Figs. 43, 45). I have obtained a few intact later cleavages, particularly in eggs left for several hours after recentrifuging before fertilizing them (Fig. 44).

The granular quarter-eggs (Fig. 46), on the other hand, although having no female nucleus, develop quite normally. The male aster can

be seen in the granules (Fig. 47), the nucleus enlarges, disappears, and the egg cleaves a little later than the control eggs (Fig. 48). This then divides into 4, 8, 16 approximately equal cells usually retaining the fertilization membrane (Figs. 49, 50).

Whether the quarter-eggs could give rise to swimming plutei will be investigated further. This part of the work was done for a short period late in September when the eggs are not in the best condition. The granular quarters went as far as the stage just before they become free-swimming and looked quite normal at that time.

The recentrifuged half-eggs which have become dumb-bell shaped (Fig. 51) retain their shape if fertilized immediately, and round up if left unfertilized just as the whole eggs do. The rounded eggs develop in the same way as before recentrifuging. The dumb-bell shaped half-eggs form a fertilization membrane following their contour and the nuclear phenomena accompanying fertilization can be clearly seen (Figs. 52–54). The first cleavage usually comes in near the junction of the clear with the granular area, giving one clear cell with oil cap and one granular cell (Fig. 55). The granular cell often precedes the clear cell in division just as it does when completely separate. The fertilization membrane usually breaks after several divisions, giving a loose mass of cells, some clear and some granular (Figs. 56–59).

Micro-dissection

The difference in the material of the white and red half-eggs can be well demonstrated by micro-dissection. When the colorless half-eggs are punctured by a needle they immediately explode, the granules and nucleus flowing out and leaving the membrane empty. When the pigmented half-eggs are punctured, there is no flow of granules, the material is quite pliable and elastic; it can be pulled out in strands which will go back again and resume a spherical form, or it can be cut in parts, each of which may round up. The stratified whole egg responds in a similar way. When the clear zone is punctured, the granules flow out. When the yolk or red layers are punctured, the material can be pulled out and released without any explosion or loss of material, and it behaves like an elastic and pliable substance. When the clear quarter-egg is punctured, it explodes immediately. When the granular quarter is punctured, the granules flow out but quite slowly.

Parthenogenesis

The question of parthenogenesis in the half- and quarter-eggs has been studied only slightly. Just (1928), and others previously, found that by treating unfertilized *Arbacia* eggs for a few seconds with

distilled water and then returning them to sea water, the eggs formed fertilization membranes and developed to the stage just before cleavage. When the half- and quarter-eggs are thus treated, they all form beautiful fertilization membranes and good ectoplasmic layers. In the centrifuged whole eggs and in colorless half-eggs, the nucleus descends toward the center of the egg just as in fertilized eggs; this then is *not* an attraction by the male pronuncleus. The nucleus enlarges and breaks just as in fertilized eggs. Astral radiations characteristic of fertilized eggs at the time of union of the pronuclei are, of course, absent, but the astral radiations from the amphiaster are later seen in the granular zone. In the pigmented half-eggs, no development further than the formation of the fertilization membrane and ectoplasmic layer has been observed, nor would it be expected since there is no nucleus of any sort. The clear quarters start to develop just as the colorless half-eggs, as indicated by the descent of the nucleus to the center. The granular quarters, like the pigmented half-eggs, show no further development after the formation of the fertilization membrane and ectoplasmic layer.

Summary

1. With strong centrifugal force and the proper medium, *Arbacia* eggs can be separated into two half-eggs, one colorless containing oil, nucleus, clear layer, fifth (granular) layer and a little yolk; the other slightly smaller containing yolk and pigment. With greater centrifugal force, the colorless half-eggs can be separated into quarter-eggs, one perfectly clear with oil and nucleus; the other, smaller, with fifth layer (granules) and yolk. All of these half- and quarter-eggs can be fertilized, form fertilization membranes and cleave.

2. Nuclear phenomena accompanying fertilization and cleavage, quite normal, can be observed with great clearness in the colorless half-eggs. Astral rays occur only where granules are present. These half-eggs cleave regularly and form swimming blastulae and plutei, normal except for color and size.

3. The pigmented half-eggs develop with only the male nucleus which divides repeatedly, usually without cell division. Some blastulae and a few plutei developed but these eggs and larvae are not very viable.

4. Whole eggs, centrifuged till dumb-bell shaped, retain their shape if fertilized immediately, even if the fertilization membrane is removed. The first cleavage in elongate eggs is usually parallel with the stratification, in spherical eggs it is usually perpendicular to it. Slipper shaped blastulae develop from the elongate eggs, and normal plutei.

5. Clear quarter-eggs begin to cleave very slowly (after 7 hours), and usually form loose clusters of cells owing to the breaking of the fertilization membrane.

6. Granular quarter-eggs develop with only the male nucleus and a little more slowly than the normal whole eggs; cleavage is quite regular but no swimming blastulae were obtained.

7. The pigmented half-eggs can be drawn out with a micro-dissection needle, and the material is pliable and elastic; the colorless half-eggs explode when punctured, pouring out granules. The clear quarter-eggs collapse immediately when punctured, and the granular quarters pour out their granules slowly.

8. All of the half- and quarter-eggs will start to develop parthenogenetically, *i.e.*, throw off a fertilization membrane, if treated with distilled water. Only those with a nucleus develop further, till just before cleavage.

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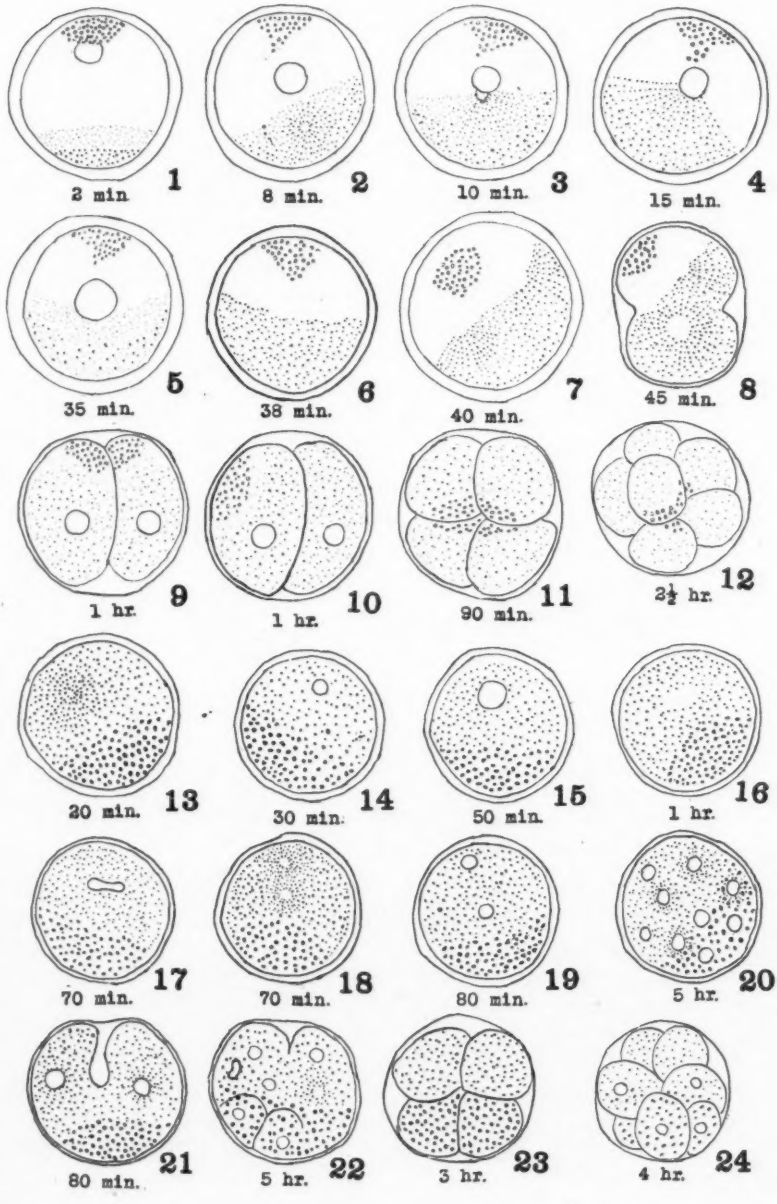
DESCRIPTION OF PLATES

The drawings have been made from living eggs entirely, and are magnified 266 X. The large solid dots represent pigment, the coarse stippling yolk granules, fine stippling the granules of the fifth layer, small circles oil drops. The times given are times after fertilization, approximate for 23° C. (controls cleave in 50 minutes).

FIGS. 1–12. Colorless half-eggs; Figs. 13–24 pigmented half-eggs; Figs. 25–35 centrifuged whole eggs; Figs. 36, 37 normal whole eggs; Figs. 38–45 clear quarter-eggs; Figs. 46–50 granular quarters; Figs. 51–59 recentrifuged colorless half-eggs.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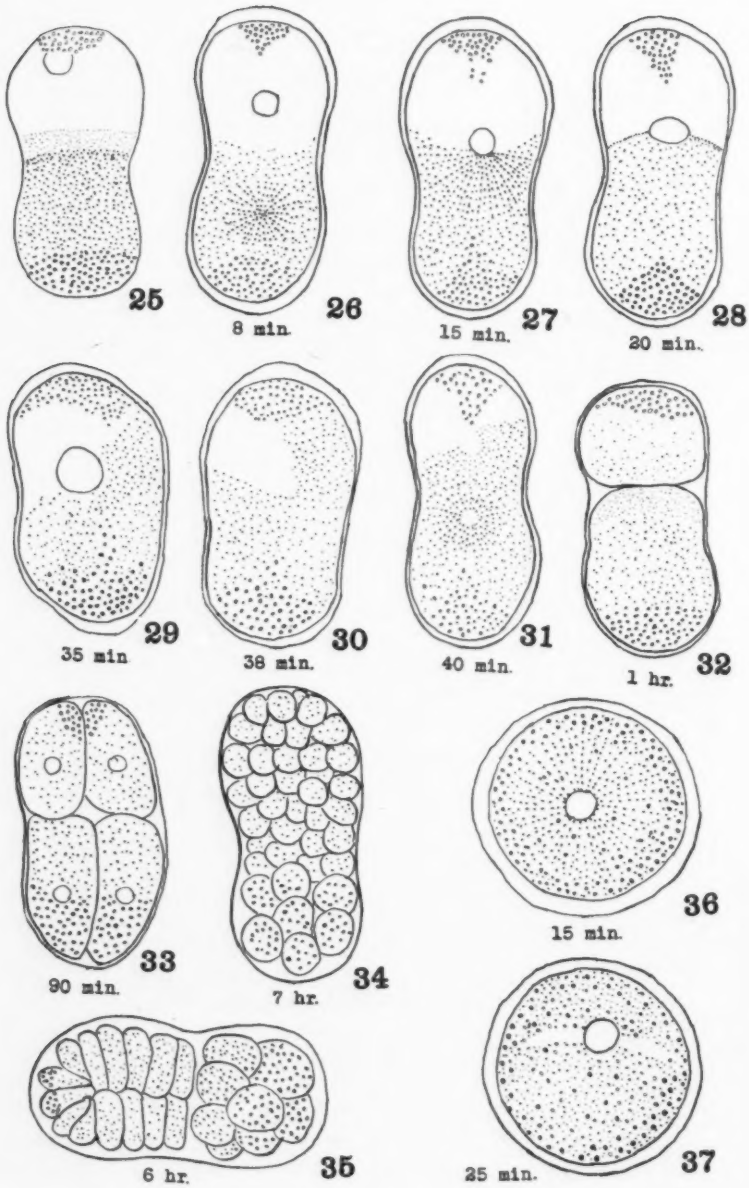
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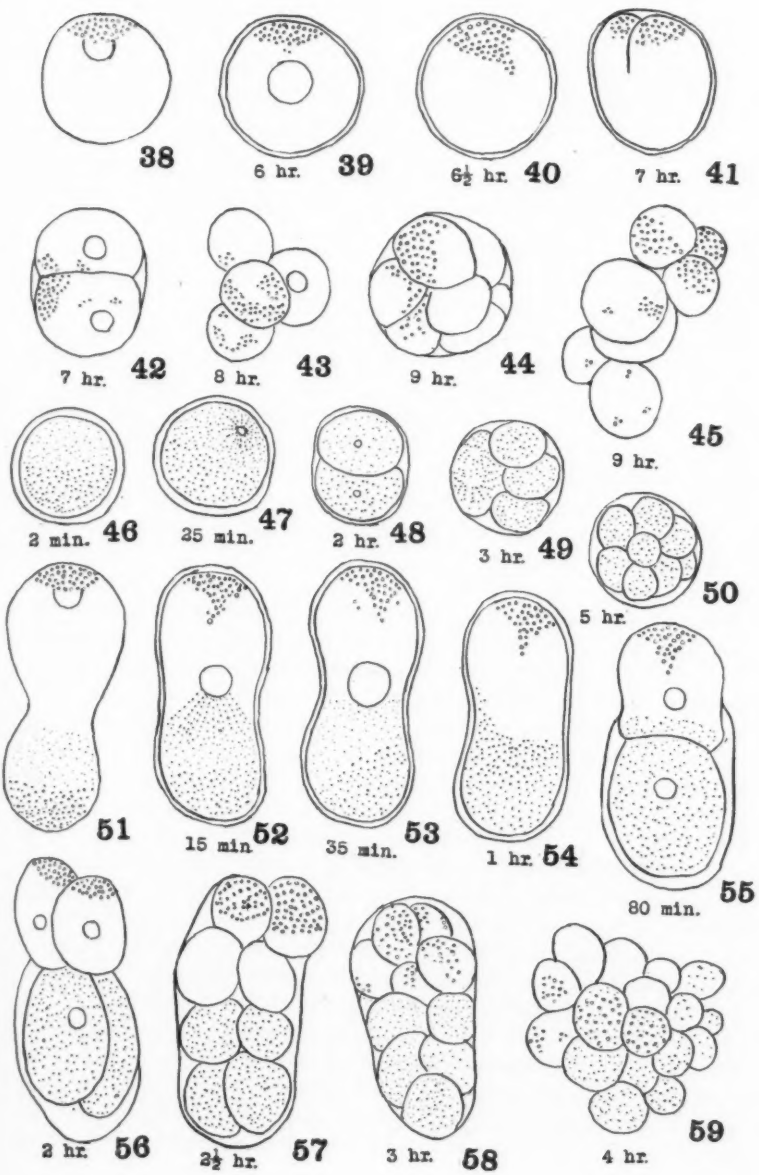
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4 hr. 59

MIGRATION OF THE PROXIMAL RETINAL PIGMENT IN THE CRAYFISH IN RELATION TO OXYGEN DEFICIENCY

RUDOLF BENNITT AND AMANDA DICKSON MERRICK

ZOÖLOGICAL LABORATORY, UNIVERSITY OF MISSOURI

The primary factors determining the position of the pigment in the proximal and distal sets of pigmented cells of the crustacean retina (Fig. 1) are light and darkness. Whether they act directly on the cells or indirectly via the circulation or the nervous system has not been definitely determined. But ever since Exner (1891) called attention to the optical importance of ensheathing the visual axis (Fig. 1, rhabdome and cone) of the ommatidium with opaque pigment in the light and of withdrawing this sheath in darkness, thereby allowing the photoreceptor (the rhabdome) to make use of light other than that which enters along its axis, most investigators have regarded this as a more or less adaptive mechanism, though of somewhat doubtful effectiveness.

It became apparent, however, that under some circumstances factors other than light and darkness might induce changes in the proximal and distal sets of pigmented cells of the crustacean retina. For example, Congdon (1907) found that temperature was such a factor in certain Crustacea. Demoll (1911) and Bennitt (1924) found that anaesthesia and death, in noctuid moths and crustaceans respectively, were always accompanied by the extreme "light-adapted" position of the retinal pigment, regardless of the surroundings. Welsh (1930*a*) recently noticed that anaesthesia had this effect on the distal retinal pigment of the shrimp *Macrobrachium*. He further observed a "diurnal rhythm" in the migrations of the distal pigment cells in the retina of this animal, even under constant illumination, and this he believed to be associated with the same sort of "metabolic periodicity" to which had been ascribed numerous other cases of periodic change—in color, luminescence, etc. Bennitt (1932*b*) observed a similar, though less extensive, diurnal rhythm in the proximal pigment cells of the retina in crayfishes which were kept for several days in total darkness. Finally, Bennitt (1924, 1932*a*) obtained evidence that under certain conditions stimulation of one eye might result in bodily changes affecting the position of the proximal pigment in the other (unstimulated) eye; whether these changes are nervous or vascular or both is still undecided.

It would seem, therefore, that in addition to the usual photic changes associated with the action of the proximal pigmented cells of the retina there is an internal mechanism of some sort whose operation may, under certain conditions, bring about changes which were at first thought to be solely photomechanical. The experience of Demoll, Bennitt, and Welsh with anaesthetics, referred to above; Arey's (1916) discovery that oxygen deficiency was associated with retraction of the

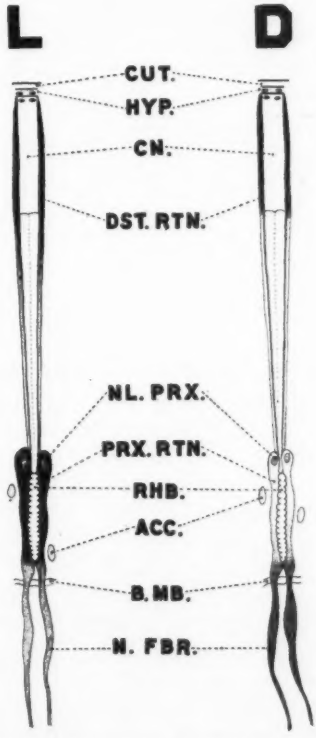


FIG. 1. Longitudinal sections of two ommatidia of *Cambarus virilis* Hagen, showing the arrangement of retinal pigment in the light-adapted (*L*) and dark-adapted (*D*) conditions. Only two of the seven proximal retinal pigment cells are shown in each ommatidium.

ACC., nuclei of accessory pigment cells; *B.MB.*, basement membrane; *CN.*, cone; *CUT.*, cuticle; *DST.RTN.*, distal retinal pigment cells; *HYP.*, cells of the hypodermis; *N.FBR.*, prolongations of the proximal retinal cells which form nerve-fibers into the optic ganglia; *NL.PRX.*, nuclei of proximal retinal pigment cells; *PRX.RTN.*, proximal retinal pigment cells; *RHB.*, rhabdome.

× 125.

retinal pigment in fishes; the work of Spaeth (1913) on the melanophores of fishes and of Uyeno (1922) on those of the frog, both of which showed distinct pigmentary changes accompanying oxygen want; and some early observations of the writers and others on the effect of overcrowding crayfish in an aquarium—all these led us to investigate the specific effect of oxygen deficiency on the action of the retinal pigment cells of the crayfish. We limited this investigation to observations on the *proximal* pigment cells, in which pigment streams distally in the light and proximally in darkness through the cytoplasm of cells of virtually fixed length (cf. Fig. 1 and Plate 1). Photomechanical movements of the distal cells have been described in the shrimp *Palaemonetes* by Welsh (1930*b*).

When a crayfish is "light-adapted" (Fig. 1, *L*; Plate 1, *L*), anaesthesia or death evoke no change in the position of the proximal pigment. When, however, the crayfish is "dark-adapted" (Fig. 1, *D*; Plate 1, *D*), death, anaesthesia, or simply overcrowding in the aquarium induce distal migration of the proximal retinal pigment even though the animal is in total darkness throughout the experiment.

General Methods

The crayfishes, *Cambarus virilis* Hagen and *C. clarkii* Girard, were killed in hot water to fix the position of the pigment, and were examined: (1) after sectioning in paraffin, or (2) after macerating for 18 hours or more in Bela Haller's fluid. The latter method was simpler, and was equally reliable for the study of proximal cells, especially since the rhabdomes and accessory pigment cells usually adhered to the dissociated proximal pigment cells.

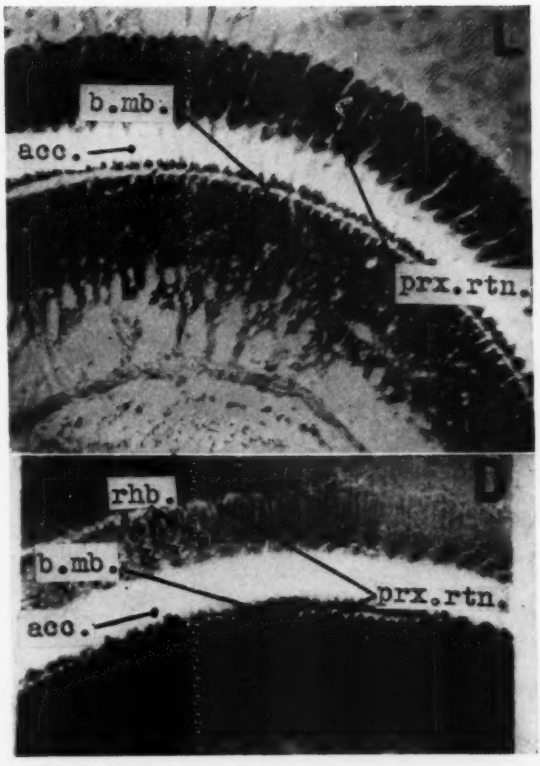
Oxygen and nitrogen were taken from commercial cylinders. Carbon dioxide was taken first from a Kipp generator, later from a commercial cylinder; no difference in results was detected.

Oxygen determinations were made by the Winkler method, modified after Kemmerer, Bovard, and Boorman (1923). Free carbon dioxide was determined by the method of Seyler (1894). The pH was determined colorimetrically by the use of the Clark and Lubs standard indicators bromthymol blue, bromcresol purple, and cresol red.

The temperature of the water was taken with each sample, and the variation was found to be within 3° C. for any given experiment.

The exact position of the proximal retinal pigment was recorded in every case, but for convenience in tabulation three general positions are used in this paper: (1) "Dark"—pigment not distal to the bases of the rhabdomes (Fig. 1, *D*; Plate 1, *D*); (2) "intermediate"—pigment extending along the rhabdomes but not distal to them; (3) "light"—pigment extending beyond the distal ends of the rhabdomes, *i.e.*,

PLATE I



Photographs of unstained sections of the compound eyes of *Cambarus virilis*, in the light-adapted (*L*) and dark-adapted (*D*) conditions. Only a part of each retina is shown, and only the proximal components of the ommatidia (cf. Fig. 1), *viz.*:

acc., accessory pigment layer; *b.mb.*, basement membrane; *prx.rtn.*, proximal retinal pigment cells, showing the nuclei at their distal ends, and extending proximally through the basement membrane; *rhb.*, rhabdomes, visible in (*D*) as the crenulated areas among the proximal retinal cells, but obscured in (*L*) by the retinal pigment. × 125.

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nearly or quite to the tips of the proximal pigment cells (Fig. 1, *L*; Plate 1, *L*).

EXPERIMENTS

General Effect of Excess Carbon Dioxide

Preliminary experiments were performed in this laboratory by Mr. L. M. Schmidt. He did not determine the oxygen content or carbon dioxide content of the water, but he found that exposure to an excess of carbon dioxide (1) inhibited proximal migration of the proximal retinal pigment when light-adapted crayfish were placed in the dark and (2) brought about distal migration of this pigment in dark-adapted animals which were kept in darkness. His method was

TABLE I

General effect of excess carbon dioxide on the position of the proximal retinal pigment —preliminary experiments.

	Number of eyes examined		
	Dark	Intermediate	Light
Controls—killed at end of experiment after 6 hours in darkness	12	2 *	0
Light-adapted animals, placed in darkness for 6 hours in dishes through which CO_2 bubbled at rate of 2 liters per hour	0	0	18
Controls—killed in darkness at beginning of exposure to CO_2	7	6 *	0
Dark-adapted animals, kept in darkness for 6 hours thereafter in dishes through which CO_2 bubbled at rate of 2 liters per hour	0	0	34

* The observed variation in pigment-position in the proximal cells of dark-adapted eyes was probably due to the fact that some of the controls were killed during the day, others at night. Bennitt (1932*b*) found evidence of diurnal variation in pigment position, even when the crayfish were in continuous darkness.

to bubble carbon dioxide (2 liters per hour) for six hours through water containing a number of crayfish, keeping other animals in running water as controls. All his animals were alive and were moving their swimmerets at the end of the six-hour period. Schmidt's results appear in Table I.

Relation between Position of Proximal Retinal Pigment, Carbon Dioxide Content, Oxygen Content, and Hydrogen Ion Concentration

Carbon dioxide was bubbled through about five inches of water in a 12 x 18 inch aquarium at the rate of 3 liters per hour. Eight or ten dark-adapted crayfish in the aquarium were prevented from rising

to the surface by a layer of sheet cork. The apparatus was kept in darkness, and at intervals of one or two hours animals were killed and water samples were taken for determination of pH, oxygen content, and carbon dioxide content.

The experiments involved 42 animals (84 eyes); 21 animals (the "dark controls") were killed at the beginning of the exposure to carbon dioxide; the other 21 were kept in water through which carbon dioxide passed, and were in darkness throughout. The results appear in Table II, in which the figures for oxygen content, carbon dioxide content, and pH represent averages for the five experiments.

TABLE II

Relation between position of the proximal pigment, oxygen content, carbon dioxide content, and pH. Carbon dioxide bubbled through the water at the rate of 3 liters per hour. Animals in darkness throughout the experiment. (*—cf. foot-note, Table I.)

	Oxygen	CO_2	pH	Number of eyes examined		
				Dark	Intermediate	Light
	cc./liter	*cc./liter*				
Dark controls	3.2	1.2	7.6	34	6 *	2 *
Continued in darkness; CO_2 for 2 hrs. at rate of 3 liters per hr.	0.8	45.0	6.0	0	6	12
Same conditions—4 hrs.	0.0	66.0	5.3	0	0	12
Same conditions—6 hrs.	0.0	124.0	5.3	0	0	12

TABLE III

Relation between position of the proximal pigment, oxygen content, carbon dioxide content, and pH. Carbon dioxide bubbled through the water at the rate of 6 liters per hour. Animals in darkness. (*—cf. foot-note, Table I.)

	Oxygen	CO_2	pH	Number of eyes examined		
				Dark	Intermediate	Light
	cc./liter	*cc./liter*				
Dark controls	3.4	35.4	7.7	2	4 *	0
Continued in darkness; CO_2 for 30 min. at rate of 6 liters per hr.	0.2	137.5	5.4	2	4	0
Same conditions—60 min.	(water-sample lost)			4	2	0
Same conditions—90 min.	0.0	277.1	4.4	0	0	6

As the experiment progressed, the carbon dioxide content in each case increased while the pH and the oxygen content decreased. In an attempt to determine whether or not there was any quantitative

relation between the rapidity of pigment migration and the rapidity of change of these physical values, carbon dioxide was next bubbled through the water at twice the former rate (*i.e.*, 6 liters per hour), and animals and water samples were removed every thirty minutes. The results appear in Table III.

All the animals in this experiment were light-adapted at the end of one and one-half hours. When carbon dioxide had been administered only half as rapidly (*i.e.*, 3 liters per hour), this condition was not attained until between three and four hours. The rapidity of migration of the proximal pigment appeared to vary in general with the rapidity of administration of carbon dioxide, though whether or not carbon dioxide itself was the active agent in the migration remained to be seen.

TABLE IV

Relation between position of the proximal pigment, oxygen content, carbon dioxide content, and pH. Nitrogen bubbled through the water at the rate of 2–3 liters per hour. Animals in darkness. (*—cf. footnote, Table I.)

	Oxygen	CO_2	pH	Number of eyes examined		
				Dark	Intermediate	Light
	cc./liter	*cc./liter*				
Dark controls (beginning)	2.1	2.7	7.5	20	6 *	10 *
Continued in darkness; N_2 for 2 hrs. at rate of 2–3 liters per hr.	0.6	8.0	7.4	0	8	20
Same conditions—4 hrs.	0.3	7.7	7.4	2	2	26
Same conditions—6 hrs.	0.0	14.2	7.3	0	0	12
Dark controls (end)	—	—	—	18	12 *	6 *

Comparing now the physical conditions, shown in Tables II and III, which obtained when distal migration was complete in all the eyes, it appeared that while the oxygen content dropped to zero in both cases, there was little uniformity in the values for either carbon dioxide content or pH. The next step was to determine whether the pigmentary response was associated with (1) surplus carbon dioxide, (2) oxygen deficiency, or (3) increase in hydrogen ion concentration.

Effect of Oxygen Deficiency with Only a Slight Increase in Carbon Dioxide Content

Instead of carbon dioxide, nitrogen was bubbled through the water at the rate of 2 to 3 liters per hour. This permitted the free oxygen to be used up as in the preceding experiments without at the same time adding artificially a large amount of carbon dioxide to that already present. The procedure was as before except that "dark controls."

kept in running water, were killed at both the beginning and the end of each exposure to nitrogen. The results of the six experiments are summarized in Table IV.

Since complete distal migration occurred in all animals killed when the CO_2-content was as low as 14.2 cc. per liter in the experiments with nitrogen, while it did not occur at much higher concentrations of carbon dioxide in previous experiments (cf. Tables II and III), it appeared that the concentration of carbon dioxide as such was not a factor affecting pigment migration in the proximal retinal cells except insofar as it might contribute to other physical changes. Moreover, since distal migration occurred in all cases at a pH as high as 7.3 in the

TABLE V

Relation between position of the proximal pigment, oxygen content, carbon dioxide content, and pH. Water acidified with HCl. The pH-range represents readings taken at the beginning and end of each experiment.

	Oxygen	CO_2	pH range	Number of eyes examined		
				Dark	Intermediate	Light
	cc./liter	*cc./liter*				
Dark controls	1.7	1.5	7.6–7.6	12	0	0
Continued in darkness; acidified water for 2 hours	(no determinations)		5.2–5.6	4	2	0
Same conditions—4 hours	0.2	5.6	5.0–6.0	8	4	2
Same conditions—2 hours	3.4	44.0	3.9–4.8	2	8	16

experiments with nitrogen, while failing to occur at a pH as low as 6.0 in the earlier experiments (cf. Tables II and III), it would seem that the hydrogen ion concentration also was not a directly contributing factor. However, additional experiments were undertaken to settle this point.

Effect of Altered Hydrogen Ion Concentration

Tap-water was acidified with HCl, and dark-adapted animals were left in it in darkness for varying periods. The "dark controls," as before, were kept in running water. The results of these experiments are listed in Table V.

Thus hydrogen ion concentration appeared to become an active factor in promoting distal migration of the proximal retinal pigment only at acidities as high as pH 3–4, and even under these conditions the effect was by no means consistent, as is indicated by the 10 eyes which failed to become light-adapted. With one exception, however, the pH of the water in all previous experiments did not go below 5, and in all

these cases distal migration had proceeded to completion. Hydrogen ion concentration, within the experimental range produced by the addition of carbon dioxide, did not affect appreciably the position of the pigment.

Discussion

It should be noted that the animals were neither dead nor even completely quiescent at the end of maximum exposures to carbon dioxide, nitrogen, or acidified water. In all cases the swimmerets were moving and in many cases the mouth-parts and other appendages as well. Check experiments showed that these animals could stand the most unfavorable conditions to which they were subjected in the experiments described in this paper, and would recover when replaced in running water.

The only one of the three factors tested which showed any consistent relation to migration of the proximal retinal pigment was oxygen content. As this decreased, the pigment began to move toward the distal position, even in darkness, and this migration was invariably completed when the oxygen tension had dropped to zero. If we are correct in supposing that in aquatic Crustacea there is a fair degree of correlation between the oxygen content of the water and the metabolic activity of the animal (as expressed by its oxygen consumption) it seems reasonable to believe that the pigmentary changes here observed are associated in some way with varying metabolic conditions. Evidence that the supposition is correct is afforded by Amberson, Mayerson, and Scott (1925). In a large number of experiments on the lobster (*Homarus*) they found a correlation between oxygen tension in the water and oxygen consumption by the animal so close as to warrant the statement that "at every instant the oxygen-consumption is directly proportional to the oxygen-tension in the sea-water at that instant" (1925, p. 175). They found an equally close correlation of the same sort in the polychaet *Nereis*, and preliminary experiments indicated a similar condition in the horse-shoe crab (*Limulus*), the blue crab (*Callinectes*), and the shrimp (*Palaemonetes*). *Palaemonetes* was able to keep its oxygen consumption constant until the oxygen content of the surrounding water had dropped to 50 per cent of saturation, but when less oxygen than this was present, its response was similar to that of the lobster.

Since distal migration of the proximal retinal pigment, though influenced primarily by the presence of light, is promoted also by low temperature, oxygen deficiency, anaesthesia, and death, it begins to look as though it might be promoted by any general factor which tends to retard metabolic activity. This idea was first presented by W. H.

Cole (1922, p. 408) with reference to the distal movement of melanophore pigment in frog tadpoles—an activity analogous in many respects to distal movement of the proximal retinal pigment in crustaceans.

The rhythmical activity of the distal and proximal pigment cells of the crustacean retina under constant conditions of light or darkness (Welsh, 1930*a*; Bennitt, 1932*b*) is a condition which scarcely admits of interpretation except on the basis of daily changes within the animal's body. It is probable that such rhythmical activity as well may ultimately be explained on the basis of variations in metabolic activity.

Summary

1. Overcrowding of crayfish in an aquarium often results in distal migration of the proximal retinal pigment while the animals remain in darkness.

2. Bubbling of carbon dioxide through the water surrounding the crayfish has the same effect.

3. When carbon dioxide is passed through the water at the rate of 3 liters per hour, distal migration of this pigment is complete at an oxygen content of 0.0 cc. per liter, a carbon dioxide content of 66.0 cc. per liter, and pH 5.3.

4. When carbon dioxide is passed through the water at the rate of 6 liters per hour, distal migration is complete at an oxygen content of 0.0 cc. per liter, a carbon dioxide content of 277.1 cc. per liter, and pH 4.4. The speed of distal migration varies in general with the speed of administration of carbon dioxide.

5. When, instead of carbon dioxide, nitrogen is passed through the water at the rate of 2–3 liters per hour, distal migration is complete at an oxygen content of 0.0 cc. per liter, a carbon dioxide content of 14.2 cc. per liter, and pH 7.3.

6. Acidification of the water with HCl, so long as the oxygen supply remains normal, produces no discernible effect on distal migration, except in the pH range 3.3–3.9, which is considerably below that involved in the preceding experiments.

7. The only factor shown to have a definite relation to distal migration in these experiments is oxygen deficiency. Distal migration is always complete when the oxygen content of the surrounding water reaches zero.

8. The various secondary factors promoting distal migration of the proximal retinal pigment—viz, low temperature, oxygen deficiency, anaesthesia, and death—all tend to retard metabolic activity. The evidence indicates that distal migration of this pigment, while influenced primarily by the presence of light, may secondarily be induced by agents which reduce the metabolic rate.

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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

III. The Applicability of Osmotic Laws to the Rate of Hemolysis in Hypotonic Solutions of Non-electrolytes

M. H. JACOBS

(From the Department of Physiology, University of Pennsylvania, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

I

Few subjects on the field of cellular physiology have received as much attention as that of osmotic hemolysis. Beginning with the observations of Hamburger (1886)—for a summary of the early literature, see Hamburger (1902)—there have appeared literally hundreds of papers dealing with this process under all conceivable conditions of health and disease, and of age, sex, species, and previous treatment of the blood. As has been pointed out in the preceding paper of this series (Jacobs and Parpart, 1931), much of this work is of very doubtful value because of the neglect of certain essential experimental precautions. Entirely apart from this defect, however, the work is disappointing in its almost complete failure to deal with the question of the rate at which such hemolytic processes occur. Unlike the published work upon other types of hemolysis (saponin, bile salts, specific sera, etc.), that upon osmotic hemolysis with few exceptions has had to do merely with the end state finally reached by the system. Information about this point, though possessing a limited practical value in medicine, is, on the whole, of comparatively little theoretical interest. So-called "fragility" studies—at least as ordinarily carried out—are concerned with the specific properties of the erythrocyte alone and they throw little light upon more fundamental problems of cell physiology. On the other hand, a study of the *rate* of osmotic hemolysis, which is closely associated with the rate of entrance of water into the cell, has obvious applications to many important general problems in the fields of osmosis, cell permeability, etc.

To all investigators who have tried in the usual way to measure the rate of osmotic hemolysis the reason for the almost complete neglect of this field in the past is clear. Osmotic hemolysis, when it goes to completion, as, for example, in distilled water or in very strongly hypotonic solutions, is such a rapid process—requiring for its entire course perhaps only a few seconds—that the ordinary methods of

studying hemolytic phenomena cannot be successfully employed with it. On the other hand, when it is sufficiently slow to permit ready measurement, especially when the end-point is some partial degree of hemolysis, the results obtained are likely to be so variable and irregular, and at first sight so generally inexplicable, that most persons who have tried to work under these conditions have soon abandoned their attempts. The author has pointed out elsewhere (Jacobs, 1927, 1928, 1931; Jacobs and Parpart, 1931) that while irregularities in the behavior of the erythrocyte can be minimized by a strict standardization of the experimental procedure, they are in part inherent in the nature of the material itself and are therefore unavoidable. The difficulty, in brief, is that factors such as temperature, pH, etc. which might be expected to affect the rate at which a given equilibrium condition is attained, have an unusually strong tendency in the erythrocyte to change at the same time the position of the equilibrium itself. Under these conditions the results are, in general, too complicated for ready analysis; and the experimenter is of necessity driven back to the other horn of the dilemma where the difficulty is with the rapidity of the process and therefore with the method rather than with the material.

Fortunately, the simple method of the author (Jacobs, 1930) for the study of hemolysis proves to be adequate for a fairly accurate determination of times of hemolysis greater than approximately 1.2 seconds and permits experiments on the rate of the process to be made under conditions where disturbing equilibrium factors are of negligible importance, namely, in distilled water and in very strongly hypotonic solutions. The method therefore opens to experimental study an important field which has heretofore been almost wholly neglected. This general field will be dealt with in the present and in several succeeding papers.

A very fundamental question, which must first be decided before other work can be undertaken with profit, is how far the rate of hemolysis in hypotonic solutions may be considered to depend upon the rate of entrance of water into the erythrocyte in accordance with simple osmotic laws. In an earlier paper by the author (Jacobs, 1927) it was tentatively assumed that the erythrocyte behaves as a simple osmometer and gives up its hemoglobin to the surrounding solution when a certain critical hemolytic volume, V_h, is reached. On this assumption equations were derived for the calculation of permeability constants for water from data on the rate of osmotic hemolysis. It was, however, emphasized in another place (Jacobs, 1931) that osmotic hemolysis is in reality a fairly complicated process involving (*a*) the entrance into

the cell of water, (*b*) the escape of hemoglobin, (*c*) the possible escape of salts and other osmotically active materials and (*d*) changes produced in various other ways in what is commonly loosely spoken of as the osmotic resistance of the cell. Only where factors *b*, *c* and *d* can be shown to be of negligible importance is it permissible to use the simple method of treating the subject previously employed; and a more critical examination of this point is therefore highly desirable.

A further need for such an examination is created by the recent work of Ponder and Saslow (1931), who have given reasons for doubting the applicability of simple osmotic laws to the erythrocyte because of the leakage from the cell during the course of at least certain types of experiments of osmotically active materials (factor *c* mentioned above). It must be admitted that in cases where such leakage occurs to any considerable extent a simple treatment of the problem is impossible. However, in view of the fact that Ponder and Saslow dealt primarily with final equilibria, arrived at in the course of a considerable time, it is by no means certain that in hemolytic experiments whose duration is only a few seconds such leakage as they have described would be a disturbing factor, though it is not impossible that it might. The question can be settled only by experiment, preferably by a comparison of the observed rates of osmotic hemolysis with those deduced according to the theory that the erythrocyte behaves as an ideal osmometer. Such a comparison will now be made.

II

The equations given in the earlier paper (Jacobs, 1927) for relating the time of hemolysis to the concentration of the medium are somewhat inconvenient because they employ the initial volume, V_0, and the hemolytic volume, V_h, of the cell. Though it has recently been shown by Ponder and Saslow (1931) that the idea of a hemolytic volume has actual experimental justification, there is, in general, a certain ambiguity in working with volumes. This is due to the fact that the volume that enters into osmotic equations is not the measured volume of the cell but rather that of the water which the cell contains. In some cases, *e.g.*, the cells of the plant *Tradescantia* (Höfler, 1917), this distinction is unimportant, but in the erythrocyte, which is loaded with hemoglobin to an extent of over 30 per cent by weight, it undoubtedly is—though, unfortunately, there is little agreement among different workers as to the actual magnitude of the true volume. For many purposes it is perhaps best to use the weight of the water in the cell as determined by chemical analysis (Van Slyke, Wu and McLean, 1923, etc.), but since under ordinary experimental conditions the

weight of the water and what might be called the osmotically effective volume of the cell, *i.e.*, that part of the total volume which takes part in osmotic changes, are approximately related in a very simple manner, it is perhaps permissible for more ready comparison with other published work in this field to retain for the present purposes the older type of osmotic equations involving volumes and concentrations.

Assuming with Hill (1930) that the water within the cell is almost entirely "free," *i.e.*, capable of taking part in osmotic equilibria, or at least that there are no marked changes during the course of the experiment in the degree of "binding" of water by cell constituents, we have the relation:

$$cV = c_0 V_0,$$

where c_0 and V_0 are the initial osmolar concentration and osmotically effective volume of the cell, respectively, and c and V are any other corresponding pair of these variables. The hemolytic volume, V_h, used in previous discussions may therefore be expressed in terms of constants and of the more convenient hemolytic concentration:

$$V_h = V_0 \frac{c_0}{c_h}.$$

Making certain necessary and probably well justified simplifying assumptions as to the nature of the diffusion of water across the membrane of the erythrocyte (see in this connection Northrop, 1927; Lucké, Hartline and McCutcheon, 1931; Jacobs and Stewart, 1932), it may be predicted that the rate at which it will enter the cell, *i.e.*, the rate of increase of the cell volume, will at any given instant be proportional to the difference in the osmotic pressures, and therefore to that of the concentrations of the internal and external solutions, and to the extent of surface of the cell.

$$\frac{dV}{dt} = kA(c - C). \tag{1}$$

The external concentration, C, may be considered to be constant since the volume of the surrounding solution is very large as compared with that of the suspended cells (approximately 1000 : 1 in these experiments). Since

$$V = V_0 \frac{c_0}{c},$$

we may write equation (1) in the form:

$$-\frac{c_0 V_0}{c^2} \frac{dc}{dt} = kA(c - C). \tag{2}$$

Fortunately in the erythrocyte, because of its peculiar biconcave shape, a considerable degree of increase in volume is possible without any change in surface. No great error will result, therefore, if A be treated as if it were constant, and this simplifying assumption permits equation (2) to be integrated at once after separating the variables; that is:

$$kAt = -c_0V_0\int \frac{dc}{c^2(c-C)}.$$

Remembering that when $t = 0$, $c = c_0$, the initial isotonic concentration for blood, we finally obtain:

$$kAt = \frac{c_0V_0}{C^2} ln \frac{cc_0 - cC}{cc_0 - c_0C} + \frac{c_0V_0}{C}\left(\frac{1}{c_0} - \frac{1}{c}\right). \tag{3}$$

For the special case where the cells swell in distilled water, and C in equation (2) is therefore equal to zero, a simpler equation results, namely,

$$kAt = \frac{c_0V_0}{2}\left(\frac{1}{c^2} - \frac{1}{c_0^2}\right). \tag{4}$$

As has already been stated, V_0, the initial osmotically effective volume of the cell, is not exactly known but is, in any case, a constant. For our present purposes, therefore, V_0 and A, which has also been treated as a constant, may be incorporated with the true permeability constant, k, to give a quantity, k', whose constancy over a range of concentrations would equally well furnish a proof of the correctness of equations (3) and (4). Since we wish to use the equations only for the point at which hemolysis occurs, namely t_h, c_h, we substitute these particular values for t and c, respectively, and also for convenience represent the ratio c_0/c_h by R, giving finally:

$$k't_h = \frac{c_0}{C^2} \ln \frac{c_0 - C}{c_0 - RC} - \frac{R-1}{C} \tag{5}$$

and

$$k't_h = \frac{1}{2c_0}(R^2 - 1). \tag{6}$$

If, therefore, the erythrocyte behaves as a simple osmometer, equations (5) and (6) should yield the same value of k' for all values of C including the value of zero when distilled water is employed.

III

To test the applicability of the theoretical equations derived in the preceding section to osmotic hemolysis, experiments were performed upon erythrocytes by the method mentioned above (Jacobs, 1930). In the present paper only a single one of the earlier experiments with

saccharose solutions will be described in detail; but it may be mentioned that essentially the same results have been obtained in a considerable number of other experiments both with this substance and with dextrose. Some of the later confirmatory experiments were performed by A. K. Parpart, whose careful assistance is gratefully acknowledged.

The blood used for the experiment here described was that of the ox, defibrinated immediately after its collection. Because of certain abnormalities that seem to develop when erythrocytes stand for some time in contact with protein-free salt solutions (Kerr, 1929), the cells were not "washed" but were kept in the approximately normal surroundings furnished by their own serum up to the instant of exposure to the hemolytic solutions. It should be noted that previous washing in isotonic non-electrolyte solutions is also contraindicated by the tendency shown by erythrocytes to become agglutinated in such

TABLE I

Times of hemolysis of ox erythrocyte in water and in saccharose solutions. R is assumed to have a value of 2.1.

Concentration	Freezing point depression	Observed time of hemolysis in seconds	k'
0.00	0.00	1.40	2.10
0.10	0.0186	1.53	2.02
0.02	0.0373	1.60	2.05
0.04	0.0747	1.65	2.26
0.06	0.1123	1.73	2.49
0.08	0.1501	1.90	2.70
0.10	0.1880	2.15	3.00
0.12	0.2261	2.50	3.55
0.13	0.2452	2.75	4.12
0.14	0.2643	3.53	4.77
0.145	0.2739	7.33	3.88

solutions. Though the procedure that was of necessity followed resulted in the introduction into the non-electrolyte solutions of slight traces of electrolytes and of proteins, these were small since the dilution of the blood employed was approximately 500 : 1 and that of the serum therefore of the order of 1000 : 1, giving a final concentration of electrolytes in the vicinity of M/6000.

With each solution four determinations of the time of hemolysis were made to the nearest tenth of a second. These figures have been averaged to give the times listed in the third column of Table I. As a rule, the individual observations in each group of four varied by only one or two-tenths of a second. Only with the highest concentrations employed, where the complicating factors previously mentioned are

present, did the observations fail to show a high degree of reproducibility. The concentrations of the solutions employed are given in the first column of the table, but for purposes of calculation the freezing point depressions were used as being more nearly proportional to the osmotic pressures than the concentrations. These were calculated by the empirical equation:

$$\Delta = 1.86C - 0.2C^2,$$

which fits very closely the data given in the "International Critical Tables" for saccharose over the range of concentrations employed.

The critical concentration for 75 per cent hemolysis, which is in practice a convenient end-point to use, was directly determined as 0.148M with a calculated freezing point depression of 0.280°. Since the freezing point depression for ox serum is in the vicinity of 0.58° (Hamburger, 1902) R, the ratio of c_0 to c_h may be taken as approximately 2.1.

From these data and from the observed rates of hemolysis, Table I has been prepared. An inspection of the figures in the first and third columns of this table, or, better, of the positions of the solid circles in Fig. 1, shows the relation between the observed times of hemolysis and the concentrations of the external solutions. It will be noted that an increase in concentration from zero (distilled water) to 0.12M or 0.13M has only a relatively slight retarding effect. The retardation then increases at a rapid rate and at a concentration of 0.148M reaches infinity. This type of curve, characterized by the relative suddenness of its final rise, has always been obtained with non-electrolytes, but, as will be shown in a later paper, not with electrolytes. How far does it agree with the theoretical predictions made by the use of equations (5) and (6)?

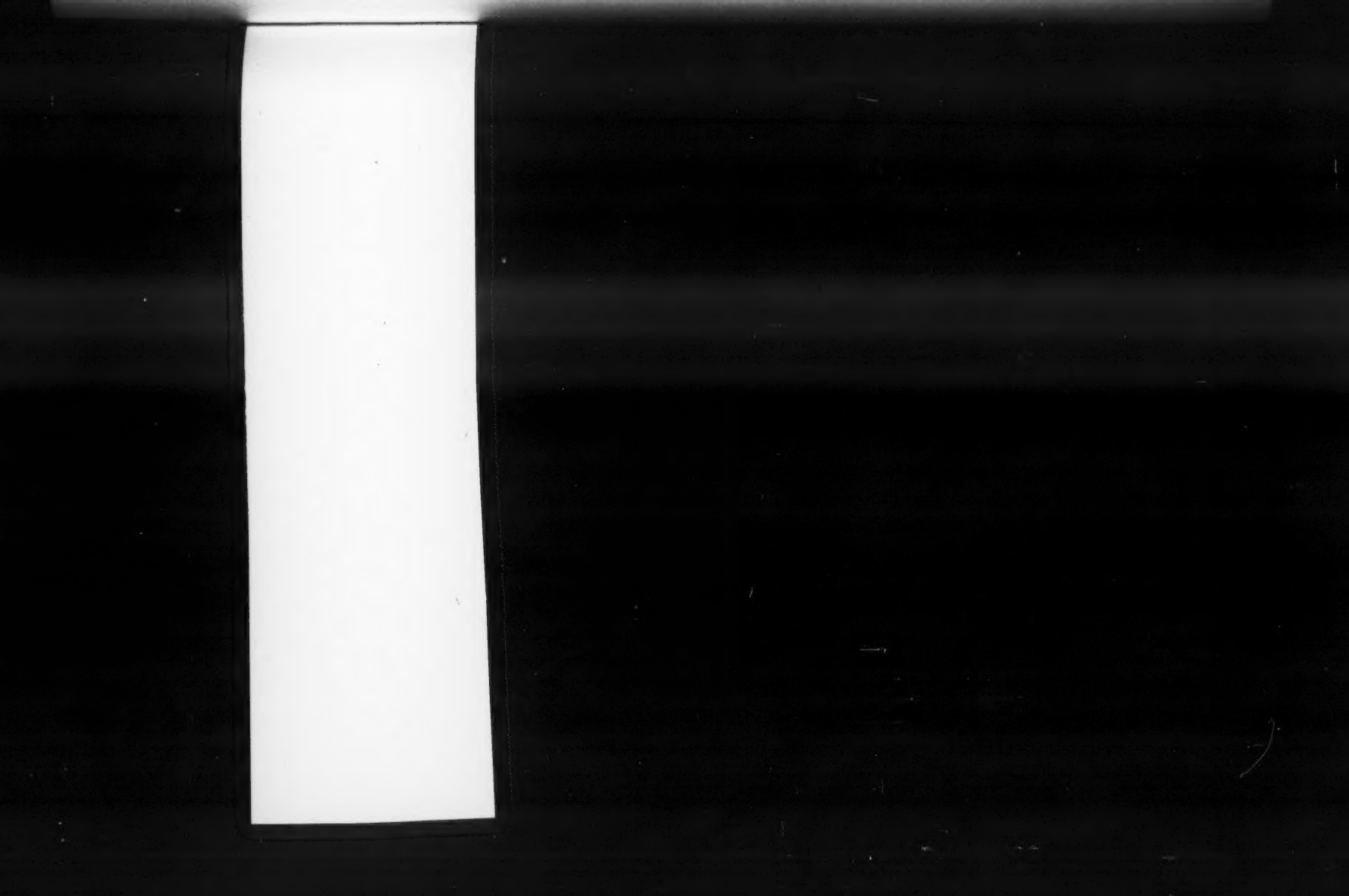
The answer to this question may be presented in two ways. In the first place, in the last column of Table I are found the calculated values of k' for the various concentrations employed. It will be noted that while there is good agreement between the values for water and for 0.01M and 0.02M saccharose, this agreement soon disappears and the last values of k' are about 100 per cent greater than the first ones. This lack of agreement is almost certainly not the result of fortuitous errors of observation, since the drift from smaller to larger values of k' is a very regular one. Evidently the predictions made from the theoretical equations depart rather widely from the observed data.

The same thing is shown still more clearly in Fig. 1 where the curve labeled 2.1 represents the times at which hemolysis ought theoretically to occur if the first few values of k' applied throughout the concen-

EDITOR'S NOTE

Line 7 on p. 184 of the article by Merkel H. Jacobs should read:

$$\Delta = 1.86C + 0.2C^2,$$



tration range instead of increasing as they do with increasing concentration. It will again be noted that the agreement between theory and observation is very poor. More specifically, the observed values increase much too slowly until the highest concentrations are reached, and then they tend to increase very rapidly. It would appear, therefore, either that the rate of hemolysis is not governed in any very simple manner by osmotic laws or that some additional factor of importance has been overlooked in making the calculations.

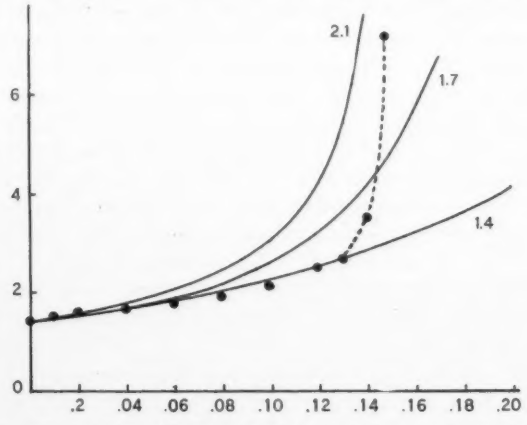


FIG. 1. Observed times for hemolysis of ox erythrocytes in distilled water and in saccharose solutions of different concentrations represented by the solid circles. Calculated times for different values of R represented by the curves. Ordinates represent times; abscissae, concentrations in mols per liter.

IV

A careful examination of the data suggests that the reason for the discrepancy between observation and theory lies not so much in the inapplicability of simple osmotic laws to the hemolytic process as in a serious error in obtaining experimentally the value of the critical hemolytic concentration, c_h. This error, in turn, is responsible for one in the value of R which appears in equations (5) and (6). An inspection of these equations, or even better, the actual substitution in them of several different values of R, all other figures remaining the same, shows that the effect of this constant on the calculated values of k' is very great and that even a small error in its determination must have serious effects. Now it might seem that of all the values entering into the calculations that of c_h, and consequently also that of R, are the most reliable, since the concentration of the solution in which a given degree of hemolysis—for example, 75 per cent—is finally

reached can be determined by direct observation with a very high degree of accuracy. But before using for purposes of calculation values of c_h and of R as so determined account must be taken of a very suspicious circumstance, namely, that the osmotic pressure of the solution of sucrose which ultimately gives 75 per cent hemolysis is very different from those of diluted serum or of NaCl having the same hemolytic effect.

For example, in four separate experiments to be described in a later paper, it was found that the unbuffered solutions of NaCl in which 75 per cent hemolysis was ultimately attained with a dilution of blood of approximately 1 : 500 had concentrations of 0.097M, 0.093M, 0.104M and 0.098M, with an average value of 0.098M. The freezing point depression of such a solution is approximately 0.340°, giving a value of R of 1.7 instead of 2.1 as determined above. In other words, erythrocytes in solutions of saccharose and, as may readily be shown, in solutions of other non-electrolytes also, have a higher resistance than in solutions of electrolytes or in diluted serum. This effect has been noted by many workers and has been variously explained. Leaving undetermined for the present the exact mechanism by which it is produced, we may accept it as a known fact and consider some of its possible consequences.

When an erythrocyte is placed in a non-electrolyte solution it possesses certain osmotic properties which are changed by exposure to its new surroundings. The critical hemolytic concentration as actually determined in such solutions is, therefore, obviously that of a cell whose properties have been modified and not that which the unaltered erythrocyte ought theoretically to show. But suppose, as seems likely, that this change is not an instantaneous one, but requires an appreciable time, say, 10 seconds, for its completion. Evidently under these conditions very rapid hemolytic processes requiring only two or three seconds would be finished before much change in the erythrocyte could occur; on the other hand, if the duration of the hemolytic process were 15 or 20 seconds, it would be sufficiently slow to permit the change—in this case an increase in the resistance of the erythrocyte—to take place. Indeed, a point would rather suddenly be reached where the increased resistance developed by the erythrocyte would be sufficient to prevent completely the hemolysis that would otherwise occur; and at this point there would be a sudden rise of the time-of-hemolysis curve to infinity.

As has been noted above, the curve expressing the observed relation between concentration and time of hemolysis shows as its most striking peculiarity a sudden rise as the highest concentrations are

approached. By far the simplest explanation of this peculiarity—indeed the only one that has so far occurred to the author—is that the increased resistance which is known to be brought about in non-electrolyte solutions does not develop instantly but requires for its appearance several seconds—almost certainly more than two or three. If this rather plausible view be accepted, then it is obviously erroneous to use the observed value of c_h in calculations involving water and very dilute solutions in which hemolysis occurs in from 1.4 to 2 or 3 seconds, *i.e.*, before the original osmotic properties of the erythrocyte have been greatly altered. Some other higher value of c_h would evidently govern the behavior of the erythrocyte in such media. Let us assume as a first approximation that the true value of c_h is that determined by the use of NaCl rather than by sugar solutions. From the figures given above, the new value of R would be in the vicinity of 1.7 (*i.e.*, 0.58 ÷ 0.340). Using this figure as preferable to the old one of 2.1 there have been calculated the new values of k' in column 2 of Table II.

TABLE II

Values of k' calculated from the data of Table I, assuming $R = 1.7$ and $R = 1.4$.

Concentration	$R^{k'} = 1.7$	$R^{k'} = 1.4$	Concentration	$R^{k'} = 1.7$	$R^{k'} = 1.4$
0.00	1.16	0.59	0.08	1.34	0.63
0.01	1.11	0.51	0.10	1.39	0.64
0.02	1.12	0.56	0.12	1.46	0.63
0.04	1.20	0.59	0.13	1.48	0.62
0.06	1.29	0.62	0.14	1.32	0.53

It is immediately apparent on examination of this table (see also the curve labeled 1.7 in Fig. 1) that the agreement between observation and theory, as indicated by the relative constancy of k', is now considerably better than before, though there is still a slow drift in the constant, which can scarcely be accounted for by experimental errors. It is to be noted, however, that the critical concentration as inferred from NaCl experiments is itself probably too low. According to Ponder and Saslow (1931), in experiments whose duration is of the order of magnitude of the time used to determine this figure (1 hour), there is a change, interpreted by them as due to an escape of salts from the erythrocyte, which is sufficient to influence the cell-volume and which would undoubtedly render hemolysis by hypotonic solutions more difficult than otherwise. If this conclusion be accepted, then the observed critical concentration, c_h, is still too low and the assumed value of R of 1.7 is too high.

Though it is impossible at present to be certain what further

correction is justified, it may be of interest to assume for the unaltered cell a value of R of 1.4. Calculations made by using this figure are given in column 3 of Table II. It will be noted that the value of k' is now almost constant, indicating an agreement of theory and observation, up to a concentration of 0.13M. This agreement is even more strikingly shown in Fig. 1 where the curve labeled 1.4 has been calculated for this value of R by means of equations (5) and (6), starting with 1.40 seconds as the time required for hemolysis in distilled water. An even better agreement could be obtained by taking a slightly lower value of R; but, in view of the simplifying assumptions used in deriving the equations, it is questionable whether the almost perfect fit that could be secured in this way has any very great significance. The important thing is that by assigning a not-improbable value to c_h (the theoretical hemolytic concentration for *the unaltered cell*), the behavior of the erythrocyte over a wide range of concentrations shows a good agreement with simple osmotic laws, and its deviation from these laws at very high concentrations can be plausibly accounted for.

V

Up to this point the increased osmotic resistance of the erythrocyte in solutions of non-electrolytes has been accepted merely as an observed fact with no attempt at an explanation. Though for present purposes it is not strictly necessary that the cause of this peculiarity of the erythrocyte should be known, it may be noted that there are not lacking a number of more or less plausible explanations which because of their general theoretical interest may now be briefly considered.

The first explanation is that non-electrolytes actually have a toughening and strengthening effect upon the cell membrane which renders the erythrocyte less susceptible to hemolysis (see for example Rhode, 1923). Although a solidifying effect of non-electrolytes upon certain gels and upon both plant and animal cells has frequently been observed (for literature upon this subject see Höber and Memmesheimer, 1923; and Höber, 1926), it seems very unlikely that this explanation is capable of accounting for such a remarkable increase in the osmotic resistance of the erythrocyte as is known to occur. In the first place, the membrane of this cell is so delicate that it probably offers little opposition under any conditions to volume changes (for some of the evidence see Jacobs, 1931). Even the much better developed membrane of the egg of *Arbacia* seems to be capable of resisting only very feebly osmotic volume changes (Lucké and McCutcheon, 1932; Harvey, 1931; Cole, 1932). It is almost inconceivable that the membrane of the erythrocyte could be so strengthened

in the absence of electrolytes as to support an excess osmotic pressure of over an atmosphere (*i.e.*, the difference in the osmotic pressures of solutions of NaCl and of saccharose, which just permit 75 per cent hemolysis to occur). Furthermore, according to Ponder and Saslow (1931) osmotic hemolysis does not necessarily involve any very appreciable stretching of the cell membrane. It seems unlikely, therefore, that a direct mechanical effect of this sort on the cell is chiefly involved.

A second possibility is somewhat more plausible. It is that in a hypotonic non-electrolyte solution there is a sufficient leakage of electrolytes from the interior of the cell to lower the internal osmotic pressure and so to reduce the amount of swelling that would otherwise occur. This explanation of the increased osmotic resistance of the erythrocyte in non-electrolyte solutions has been accepted, among others, by Bang (1909) and by Ponder and Saslow (1931). The last named authors suppose "that leakage is greater in glucose than in NaCl and that this accounts for the critical volume being reached in a solution of glucose which is more hypotonic than one of NaCl." They cite in support of this view the direct chemical evidence obtained by Kerr (1929) that in solutions deficient in blood proteins there may be an escape of potassium from and an entrance of sodium into the cell. Bang (1909) also gives references to earlier work indicating a passage of electrolytes from the erythrocyte into non-electrolyte solutions, while Joel (1915) has studied this process and the influence upon it of narcotics, by an electrical conductivity method. That electrolytes may escape from the erythrocyte into non-electrolyte solutions may therefore be regarded as a well-established fact.

It is very questionable, however, whether such an escape of electrolytes, which is probably associated with a loss of the normal permeability of the cell to cations, is capable of accounting for the very rapid rise of osmotic resistance that occurs in the present experiments. From the data shown graphically in Fig. 1 it would seem that a marked increase in resistance in non-electrolyte solutions must occur in less than five seconds, while even under the conditions of their experiments, Ponder and Saslow (1931) state that "the equilibrium volumes are attained within a minute and are maintained for hours." Since the diffusion of cations reported by Kerr (1929) is a process that seems to extend over hours, while the rise in conductivity studied by Joel went on gradually and steadily throughout experiments also lasting up to several hours, it would seem that some factor other than an outward leakage of salts (*i.e.*, of both anions and cations) is involved in the case of very rapid changes. The factor that immediately suggests itself is a

new ionic equilibrium of some sort, attained primarily by the movement of anions such as is known to occur readily in normal erythrocytes. How far the results of Ponder and Saslow with electrolyte solutions can be so explained cannot at present be stated with certainty; but, at all events, it seems likely that the extremely rapid increase in the osmotic resistance of erythrocytes that takes place in non-electrolyte solutions is to be accounted for in this way.

This view is supported by the work of Netter (1928), who has pointed out that theoretically ionic exchanges should by no means be absent between erythrocytes and a surrounding isotonic solution of a non-electrolyte, but that anions from the erythrocytes would tend to be exchanged for OH' ions from the aqueous solution in such a way as to make the interior of the cells more alkaline. This principle has been put to practical use by Bruch and Netter (1930) in obtaining various desired relations between external and internal pH values. Now it is known, especially from the work of Warburg (1922) and of Van Slyke, Wu and McLean (1923), that a change in reaction within the erythrocyte has important osmotic consequences. The osmotic pressure of a given amount of base bound by hemoglobin is considerably lower than that of the same amount of base bound by, for example, carbonic acid. If the compound of base with hemoglobin be represented as B_nHb, the osmotic pressure of this compound when completely dissociated would be to that of the same amount of base combined with carbonic acid as $(n + 1) : 2n$. Anything, therefore, which causes a shift of base from hemoglobin to carbonic acid should increase the internal osmotic pressure of the cell and cause the latter to swell; anything that causes a shift in the reverse direction should have the opposite effect. Since the new ionic equilibrium attained in non-electrolyte solutions is obviously of the latter nature, it should, without any escape of salts as such, result in a lowering of the internal osmotic pressure, and so raise the resistance of the cell to hemolysis by hypotonic solutions.

If this view of the mechanism of the increase in the osmotic resistance of the erythrocyte in non-electrolyte solutions be correct, then it ought to be possible even in solutions of electrolytes to produce the same characteristically sudden rise in the time-of-hemolysis: concentration curves described above by slightly increasing the alkalinity of the medium with a trace of NaOH or NH_4OH. This is, in fact, the case, as will be shown in a forthcoming paper by the author in collaboration with A. K. Parpart. For this reason, as well as for the others mentioned above, it seems likely that while a leakage of salts (*i.e.*, of both anions and cations) is by no means excluded as a possible factor of importance in experiments of longer duration, the factor

chiefly concerned in producing increased resistance under the conditions of these experiments is an entirely normal shift of anions alone, which because of certain peculiarities of hemoglobin, is secondarily responsible for a change in the internal osmotic pressure of the cell.

VI

It is a matter of some interest to determine the value of the true permeability constant, k, of equations (1), (3), and (4). This is a measure of the amount of water that would cross the membrane of the erythrocyte through unit area in unit time with unit difference in osmotic pressure between the cell and the surrounding solution. Such a constant would be useful for comparing the permeability to water of different cells, or of the same cell under different conditions (see Jacobs 1927, 1931).

The true permeability constant, k, is found from k' of equations (5) and (6), by multiplying by V_0, the initial osmotically effective volume of the cell, which may be measured in cubic micra, and dividing by A, the area of the cell, which is conveniently expressed in square micra. Unfortunately, both because of the small size of the erythrocyte and its peculiar shape, it is difficult to measure its surface with very great accuracy. This difficulty is well illustrated by the fact that the estimates made by different investigators for the erythrocytes of the same species of mammals may differ by as much as 50 per cent or more. Furthermore, even though the total volume of the cell can be fairly accurately determined by several methods (Ponder and Saslow, 1930) there has in the past been much uncertainty in calculating from it the osmotically effective volume, though it seems likely that this is in reality not very different from that of the total water contained in the cell (Hill, 1930). Finally, there is the serious difficulty, mentioned above, that because of uncertainty as to the value of the theoretical critical hemolytic concentration for the unaltered erythrocyte the value of k' itself is subject to a considerable error. It is evident, therefore, that the most that can be expected at present is to obtain the order of magnitude of the true permeability constant; but even this would be of considerable value.

For the ox erythrocytes chiefly used in these experiments we may take as a first approximation a value of R of 1.7. This value is obtained from the observed hemolytic concentration of NaCl rather than from that of saccharose, since, as has been mentioned above, the normal osmotic properties of the erythrocyte are very quickly changed in non-electrolyte solutions. Indeed, even the value selected is probably somewhat too high, but in the absence of more complete information it is a convenient one to use.

As to the necessary constants for the cell itself, the few published estimates are not in very good agreement. Probably the best available value for the volume of the ox erythrocyte is that given by Ponder and Saslow (1930) of 44 cubic micra. No estimates of surfaces are given in this paper, but in an earlier publication Ponder (1924) has given 37 cubic micra and 69 square micra as the values of the volume and surface, respectively, of the erythrocyte of the calf. If it be assumed that the shape of the somewhat larger cell is exactly the same as that of the smaller one, then its surface would be $69 \times \left(\frac{44}{37}\right)^{2/3}$ or 77 square micra, and this value will here be used.

In the absence of accurate chemical analyses of ox erythrocytes, it may tentatively, though perhaps somewhat questionably, be assumed that they contain the same percentage of water as those of man and that all of this water is "free." Taking an average of the figures given by Henderson (1928) for cc. of water in 1 liter of cells for three normal human individuals, and applying the same percentage to the ox erythrocyte whose total value is 44 cubic micra, we have for the initial effective osmotic volume, V_0, 0.69×44 or approximately 30 cubic micra. Remembering that the time for 75 per cent hemolysis in water is 1.4 seconds, we have all the data necessary to calculate from equation (6) the value of k.

$$k = 0.08 \times \frac{1}{1.4} \times \frac{1}{1.16} \times \frac{30}{77} \times [(1.7)^2 - 1] = 0.036.$$

The factor 0.08 has been introduced to change freezing point depressions in degrees centigrade into osmotic pressures in atmospheres. Expressed in words, the value of k so obtained means that with a difference in osmotic pressure of one atmosphere between the cell and its surroundings water should theoretically pass through each square micron of the cell surface at the rate of 0.042 cubic micra per second or of 2.2 cubic micra per minute.

In an earlier paper (Jacobs, 1927) the value of k was estimated to be of the order of 3.0 for human erythrocytes when the unit of time was taken as one minute. Since the details of the calculation were not given at that time, it may be worth while to present here an additional typical set of figures, emphasizing at the same time the fact that only the general order of magnitude of the results obtained from them is significant. In this particular case c_h in terms of freezing point depressions was found to be 0.232, while c_0 was taken as 0.56, giving a value of R of 2.4. The observed time of hemolysis was 2.4 seconds. Following Emmons (1927) the volume of the human erythrocyte may

be taken as 88 cubic micra (of which 0.69×88 or 61 cubic micra represents the true osmotic volume) and the surface as 145 square micra. We have, therefore:

$$k = 0.08 \times \frac{1}{2.4} \times \frac{1}{1.12} \times \frac{61}{145} \times [(2.4)^2 - 1] = 0.060$$

or, if the unit of time be taken as the minute, 60 times this value or 3.6.

In view of the large unavoidable errors in these calculations, due especially to uncertainty as to the exact value of R, it is questionable whether this apparently greater permeability to water of the human erythrocyte as compared with that of the ox is significant. In any case, the difference in the permeabilities of the two kinds of erythrocytes to water is far less than is that to glycerol (Jacobs, 1927, 1931) or to erythritol and to certain ions (Mond and Gertz, 1929).

Summary

1. Equations are derived for predicting the relation between the time required for osmotic hemolysis and the concentration of a surrounding hypotonic medium.

2. It is shown that when allowance is made for certain known peculiarities of the erythrocyte the rate of hemolysis is, on the whole, in fairly good agreement with osmotic laws.

3. Reasons are given for believing that the increased osmotic resistance of the erythrocyte that develops within a few seconds in solutions of non-electrolytes is not caused by a leakage of salts from the cell but rather by a changed ionic equilibrium in which the normal impermeability of the cell to cations need not be lost.

4. Rough quantitative estimates are made of the permeability of the erythrocytes of the ox and of man to water.

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LOCOMOTOR ORGANS OF ECHINARACHNIUS PARMA

GEORGE H. PARKER AND MARGARET A. VAN ALSTYNE

MARINE BIOLOGICAL LABORATORY, WOODS HOLE

The method of locomotion in the sand-dollar, *Echinarachnius parma* (Lam.), is not clearly understood. MacBride in the Cambridge Natural History (1906, p. 548) states that "The locomotor tube-feet (in *Echinarachnius*) are very small and feeble compared with those of *Echinus esculentus*, but this is comprehensible when it is recollected how little resistance the yielding sand would offer to the pull of a powerful tube-foot like that of the Regular Urchins, for in order to move the creature through the sand a multitude of feeble pulls distributed all over its surface is necessary, and the locomotor tube-feet are exactly fitted, both as to size and number, for this object." It is quite plain from this quotation that MacBride is of opinion that locomotion in the sand-dollar is accomplished by the tube-feet. In conversation with a well-known specialist in this group of animals, the senior author was informed that *Echinarachnius* possesses no tube-feet at all excepting those that are modified for gills. When experts on the echinoderms differ so widely on a simple question of fact the obvious step is to reinvestigate the subject. The present studies were carried out at the Marine Biological Laboratory, Woods Hole, where an abundance of living specimens of *Echinarachnius* was available.

The locomotion of the sand-dollar has already been reported upon in an earlier paper by the senior author (Parker, 1927). In this publication, it was shown that *Echinarachnius* exhibits two types of locomotion, rotational and rectilinear. In the second type the sand-dollar creeps forward on an axis corresponding to that of its structure. This axis is represented by a straight line passing through the animal and intercepting mouth and anus. Although the animal is in general radially symmetrical, this axis divides it into right and left halves that are structurally significant in its locomotion. In addition to its rectilinear and rotational movements, the sand-dollar can right itself after having been thrown on its back. Such righting reactions also call for an appropriate locomotor mechanism. The question to be discussed in these pages is what structures are concerned with these several types of locomotion. Three classes of organs may be suspected of having to do with such activities. They are the integumentary cilia, the tube-feet (if present), and the spines.

1. *Integumentary Cilia.*—Integumentary cilia have been identified with certainty only on the spines. In *Echinarachnius* the spines are of two kinds, long and short. These two kinds occur on both the oral and the aboral surfaces. The short spines are provided with rounded tops and their cilia occur not only on the tops but also to some extent on their sides. In the long spines the cilia are present as elongated bands on the sides of the spines from the base to near the top. The ends of the long spines are devoid of cilia. It is questionable if any cilia occur on the general surface of the integument, though it is possible that they may be found in the ambulacral grooves. Gislén (1924, p. 247) was unable to demonstrate to his satisfaction the presence of cilia in the ambulacral grooves of the allied *Arachnoides* and *Astriclypeus*.

The direction in which these cilia beat can be determined by the flow of carmine particles discharged on the surface of the sand-dollar. On the aboral face, the ciliary currents flow from the center of the test outward toward the periphery on essentially radial lines anteriorly and posteriorly, but on somewhat curved lines right and left from the axis of locomotion. In these lateral regions the direction of flow is outward and backward. On the oral surface the ciliary currents are all from anterior to posterior, no part of the surface exhibiting currents which run toward the anterior edge. In all cases both oral and aboral, these currents are so weak that they are incapable of moving even small sand particles that happen to fall among them. There is therefore no reason to suppose that the ciliary currents are concerned with locomotion. They function in all probability in the respiratory exchange of water next the animal's body and probably remove from the skin accumulations of very light silt and other dirt.

2. *Ambulacral Feet.*—In a living *Echinarachnius* a continuous fringe of active ambulacral feet can be seen around the whole edge of the test where they are to be observed in incessant activity between the numerous spines. When fully extended, they are two to three times the length of the longer spines. In contraction they draw back close to the general surface of the animal. Each foot is provided with a well-developed terminal sucker deep pink in color. Ambulacral feet essentially like those along the edge may be identified on both the oral and aboral surfaces. Here in consequence of their deep color and characteristic arrangement they give rise to a pattern which in the living animal is often of striking appearance and symmetry. On the oral surface (Fig. 1) they extend as five somewhat irregular bands along the lines of the ambulacral grooves. As these bands approach the edge of the test they widen out till on the very edge they unite to form the continuous fringe already mentioned. On the aboral surface

(Fig. 2) five well-defined double ambulacral bands of tube-feet extend from near the center of the test to its edge. Each double band passes between the two halves of a respiratory rosette. A few tube-feet are often to be seen among the gills on the outer edges of the rosettes. As the double bands approach the periphery of the test they are supplemented on each side by double series of three or more large spots, containing many tube-feet. The double bands and the supplemental spots as they approach the margin expand to such a degree that they form the continuous edging of tube-feet already described. In general the tube-feet are especially numerous and long on the edge of the test, and fewer and shorter toward the center. The shortest and least active feet occur in the ambulacral grooves. Except in the

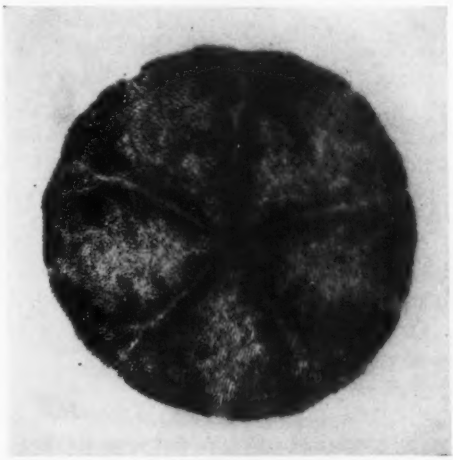

FIG. 1. Oral view of a living *Echinarachnius parma*. The dark areas beginning at the mouth and following the ambulacral grooves to the edge of the test near which they expand broadly and somewhat irregularly are the areas covered with ambulacral feet.

regions previously noted we have found no ambulacral feet in *Echinarachnius*.

When a sand-dollar moves forward on the sand in process of burrowing, the tube-feet of the anterior edge pull the grains of sand in toward the center of the animal, particularly over the anterior half of the periphery. The action of the feet is not vigorous nor well coördinated. Those along the advancing edge are most active and succeed in piling the sand into a low mound on the front of the animal. In moving forward, the animal pushes itself under this mound and the

tube-feet help thus to cover the aboral surface. It was difficult to determine the direction of pull of the feet in the middle of the oral side. When a sand-dollar was placed in a glass vessel and studied from below by means of a low-power microscope, the feet were seen to move less regularly than those along the edge of the test. In order to observe the movements of such feet, it was necessary to reduce the sand on the bottom of the glass to a very thin layer. This was unfavorable for the movements of the animal and may have been the occasion of the irregular movements seen under these circumstances. Possibly in deep sand the more centrally located feet would have shown a very different

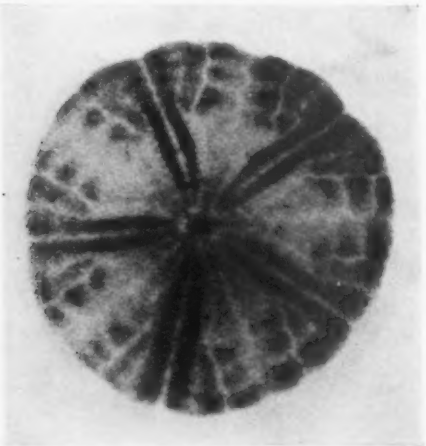

FIG. 2. Aboral view of a living *Echinarachnius parma*. The five pairs of radial dark bands and the marginal and submarginal dark spots are the areas covered with ambulacral feet.

type of movement. The pulls of the feet on the posterior portion of the test were toward the mouth, but they were feeble and less coördinated than those of the anterior edge.

There is thus no doubt of the presence of tube-feet on *Echinarachnius*, but it is improbable that the forward locomotion of this animal is in any essential way dependent upon them. In this respect our results fail to support the opinion already quoted from MacBride. The only part of *Echinarachnius* in which the tube-feet exhibit well coördinated and vigorous activity is along the anterior edge of the test, but even here their number and distribution is such that they cannot be regarded as more than subsidiary organs in the locomotion of this animal.

3. *Spines.*—The test of *Echinarachnius* is covered thickly with two kinds of spines, long and short. The only parts of the surface that lack spines are the troughs of the ambulacral grooves on the oral surface. The spines are longest near the anterior oral edge and diminish somewhat in size as one passes posteriorly from this region. As one approaches the posterior edge larger spines are again met with, but these posterior spines never reach in size those of the anterior margin. On the aboral surface the spines for the most part point toward the margin of the test, hence radially. On the oral surface the arrangement is less simple, but on both surfaces the spines exhibit a bilateral distribution in relation to the axis of locomotion. This arrangement of the spines has already been figured (Parker, 1927, Fig. 1). When a sand-dollar is placed on submerged sand, it will push forward into the sand until in 10 to 20 minutes it has covered itself completely. This operation involves forward locomotion through about ten centimeters of distance. After the sand-dollar has become covered it usually progresses less rapidly and may in fact stop moving altogether. As the animal moves forward in burying itself, the sand, as already stated, piles up over the anterior edge and gradually spreads over the aboral surface to the complete disappearance of the animal. This is due particularly to the forward movement of the animal as a whole and partly to the pull of the ambulacral feet. When sand is poured on the central part of the aboral surface of an animal submerged in water, it is spread over that surface in radial directions and this spread is dependent upon the movement of the spines of the surface.

When an animal in process of burying itself is observed through very shallow sand from the under side, the spines are seen to exhibit a striking activity. Waves of spine movement begin along the anterior border and sweep backward over the aboral surface of the animal toward the mouth and along the lateral edges of the test to the posterior region. These waves pass in one direction only. They are the result of coördinated movements of the spines, each one of which carries out a relatively complex stroke. Apparently the individual spine swings on its facet from anterior to posterior in a vigorous vertical stroke. It returns to its initial position not by a simple reversal of this movement but by a sidewise swing in a plane near that of the general surface of the test until it has reached its starting point, when a second vertical stroke takes place followed by a lateral recovery. This can be demonstrated in part by placing an inverted sand-dollar in water shallow enough barely to cover its recumbent spines. Under such circumstances waves of spine movements can be seen passing over the anterior oral surface, and the tips of the spines as they cut the water in

these waves appear always to be moving posteriorly. No spine tips in the return stroke are seen, for this is accomplished entirely under the water. These coördinated movements of the spines on the oral surface are the occasion of the animal's locomotion. Forward progress, burrowing, and righting movements of the sand-dollar are to be explained by the vigorous and well coördinated movements of these particular spines. The longest and most active spines of the anterior part of the oral side are the chief means to this end. The ambulacral feet are at best only a weak supplement to these movements.

Summary

1. The integumentary cilia in *Echinarachnius* cover the tips of the short spines and the sides of the long ones. On the oral surface they beat radially; on the aboral they beat from anterior to posterior. They play no essential part in the locomotion of the animal, but are probably concerned with feeding, with cleaning the outer surface, and with the respiratory currents.

2. The ambulacral feet form five complicated radial bands on the oral and the aboral sides of the test and a complete marginal fringe. Their tips are deep pink and provided with suckers. They are significant in locomotion to only a limited extent in that on the anterior edge of the test they pile up the sand on the aboral surface.

3. Spines cover the oral and aboral surfaces. They are of two types, long and short. They are best developed over the anterior portion of the oral surface where their distribution exhibits bilateral symmetry in relation to the axis of locomotion. In this region waves of coördinated spine movement pass from the anterior edge of the test posteriorly. In these waves each spine makes a vigorous posterior thrust in a vertical plane and an unimpeded recovery in a plane more nearly lateral. Forward locomotion, burrowing, and righting are types of motion dependent primarily on these spine movements.

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STROMBIDIUM CALKINSI, A NEW THIGMOTACTIC SPECIES

E. FAURÉ-FREMIET

Collège de France, Paris

Many ciliated Infusoria belonging to different groups can settle in a more or less temporary manner by means of their ciliated apparatus; this property is connected with some structural particularity, for example in Infusoria of the family Ancistridae and also in *Ancystropodium Maupasi*, an hypotrichous ciliate. Almost all the oligotrichous Infusoria of the family Halteriidae are planktonic species; yet some of them present occasionally thigmotactic properties and can stick or slide on the surface of solid bodies. The species of the genus *Tontonia* use, in this case, their curious caudal process lined by some small cilia; on the other hand, *Strombidium urceolare* Stein uses, according to Maupas, three long cirri located on the left side of the peristome; these cirri are fringed at their free ends whereby the Infusoria are temporarily fixed.

W. v. Buddenbrock (1922) has described under the name of *Strombidium clavellinae* a small species found in Heligoland which can either swim freely or slide on the mantle's surface of *Clavellina lepadiformis* with which this infusorian is an habitual commensal; it can also fix itself at the bottom of a dish. The fixing organ is here once more an apparatus of ciliated origin, made by four thin membranelles belonging to the left side of the adoral zone but different from the others in a considerable lengthening and in the fringed structure of their thinner ends. During the summer of 1929, I observed at Woods Hole another species of thigmotactic *Strombidium* able to fix itself temporarily by a ciliated apparatus: I will describe it under the name of *Strombidium Calkinsi*, sp. nov.

Strombidium Calkinsi is a species very nearly related to *S. sulcatum* Clap. and Lachm. and to *S. lagenula*, differing, however, from these by the presence of two long dorsal membranelles independent of the peristomal zone, the ends of which can stick on solid bodies. This infusorian measures about 40 μ in length; its body is irregularly ovoid with a great antero-posterior axis; the basal region, hemispheric, is bounded by a transversal depression; the ventral peristomal groove is lined on the right by a vertical lip extending itself on the anterior side by a so-called semicircular collar which turns dorsally toward the left.

The adoral sinistral zone takes its origin in the gullet at the bottom of the peristomal groove; this zone surrounds the collar with fourteen great membranelles. The peristomal groove, more developed than in *S. sulcatum* and less deep than in *S. lagenula*, extends toward the middle of the ventral face.

Just as in the two previous species, the posterior pole is indicated neatly not only by a transversal furrow but also by a refractive outline; this is due probably to the presence of a cuticle thicker than on the other parts of the body. A conical bundle of intracytoplasmic radiating rods fills the basal part of the body and clearly delineates the annular furrow; the significance of this formation, which exists in almost all species of *Strombidium*, is much discussed. There is no

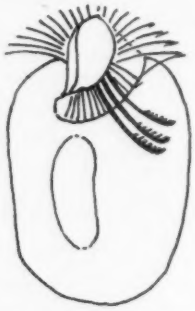

FIG. 1. *Strombidium urceolare* Stein from an original sketch of Maupas, showing "la disposition et la forme de ses longs cirres pectinés adaptés spécialement à la fixation" (Letter of Maupas, L. XI, 1907).

evidence in *S. Calkinsi* that the rods can act as trichocysts, but they can be compared to skeletal elements.

The ovoid nucleus lies in the body in the posterior region above the bundle of rods; the cytoplasm is hyaline, clear, enclosing various digestive vacuoles, and contains some granulations which are refractive and others which seemingly correspond to mitochondria.

The fixatory apparatus is not very easy to study when the infusorian is on the upper part of the slide. It is better to put the slide in a little dish and to look for Infusoria which are fixed on the edge and are, in this manner, seen in profile, as shown in Fig. 2.

This apparatus is constituted by two dorsal membranelles nearly as long as the body, measuring 35 to 40 μ in length and 7 to 8 μ in breadth. They appear delicately striated longitudinally and the coalescent cilia, which constitute these membranelles, separate more or less from each other at the adhesive distal extremity.

The insertion-lines of the two membranelles are rather near one another and produce two wrinkles obliquely bent from left to right, on the body's anterior dorsal side. From this point, the two membranelles spread, generally forming an angle more or less obtuse so that the points of fixation on the support can be slightly separated from each other.

The infusorian, when fixed, shows its ventral side up; the anterior dorsal membranelle is approximately perpendicular to the axis of the

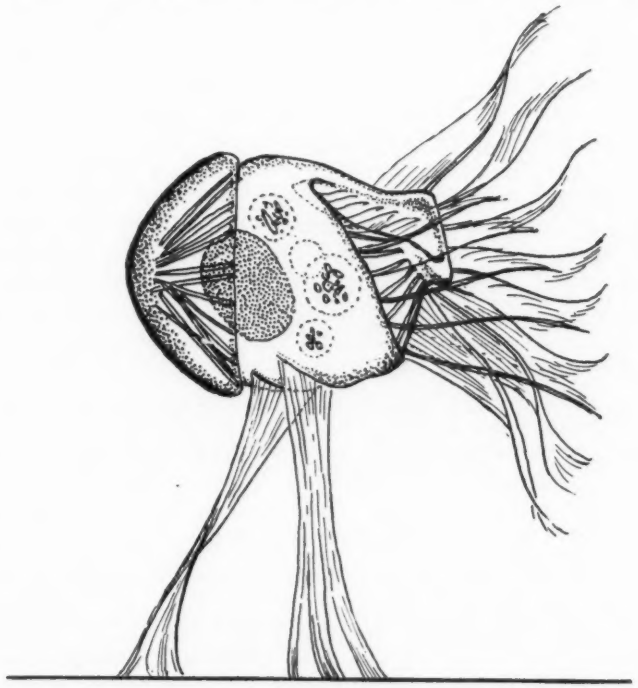

FIG. 2. *Strombidium Calkinsi* in fixed condition seen from the left side.

body and therefore to the solid support. The dorsal posterior membranelle stretches obliquely behind and often seems twisted. There is no evidence of a contractile property of the membranelles. In this fixed position the adoral zone draws around the collar half a circle perpendicular to the great axis of the infusorian's body. The vibrations of its strong membranelles produce a fluid current which flows in the ventral groove of the fixed individual. From time to

time, the reaction to the current shakes the infusorian, thus showing the elasticity of the two attaching dorsal membranelles.

At times, the infusorian seems to be walking on the solid surface like an hypotrichous infusorian, the two membranelles moving one after the other, but with no regular rhythm.

More often the *Strombidium* unfastens all at once and swims hastily, drawn forward by movement of the adoral zone. In this case the two dorsal membranelles bend along the body, showing a crumpled aspect.

It is of consequence to point out that the two dorsal attaching membranelles of *Strombidium Calkinsi* are absolutely independent of the ciliary adoral apparatus. The position of their insertion lines shows that they belong to a somatic ciliature, generally absent in the oligotrichous Infusoria, but which can reappear more or less modified, either in the form of small, short, faintly mobile cilia, inserted on the longitudinal lines, or else as a more or less developed "ciliary residual field," the presence of which I have indicated in various species of Tintinnidae. Some true cultures have developed on the surface of the slides which were put in tanks filled with running sea water.

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STUDIES ON THE CHEMICAL NEEDS OF AMŒBA PROTEUS: A CULTURE METHOD

WILLIAM F. HAHNERT[1]

(*From the Mount Desert Island Biological Laboratory, Maine and the Zoölogical Laboratory of the Johns Hopkins University*)

INTRODUCTION

The chemical needs of plants have been fairly well worked out by the culture method, but knowledge regarding the needs of animals is very inadequate. This is largely due to the fact that plants can be grown in synthetic solutions containing only inorganic salts, while animals require in addition some organic material. For example, *Amœba proteus* feeds on *Chilomonas paramecium; Chilomonas* in turn requires some organic nutrient.

In the culture of protozoa, the organic nutrient has usually been added in the form of timothy hay or grain. These substances, however, contain a considerable amount of physiologically active salts, which diffuse out into the culture and alter it in an unknown way. Under these conditions, since the kind and concentration of chemical elements are not known and do not remain constant, it is difficult to ascertain the kind and number and relative amount of elements necessary for the maintenance and growth of animal protoplasm. By a method to be described below, variation in the salt composition of the medium is fairly accurately controlled, thereby making it possible to ascertain the relative importance of individual elements in rhizopod protoplasm.

METHODS AND RESULTS

Amœba proteus (Leidy) was used in all the observations made. It was derived from stock cultures made by adding a grain of rye to a mixture of half-spring-half-distilled water in finger bowls with subsequent inoculation with amœbæ and *Chilomonas*.

Two series of experiments were performed in which variation in the salt composition of the medium was controlled. These experiments were made as follows: Five balanced salt solutions[2] were prepared as

[1] The author is indebted to Professor S. O. Mast for helpful suggestions and valuable criticisms, especially in the preparation of the manuscript, and to the Research Corporation for financial aid.

[2] Kahlbaum (analysis grade) chemicals (except Merck's blue label $Ca_3(PO_4)_2$) and water redistilled in pyrex glass were used in all solutions. According to recent investigations by Williams and Jacobs (1931), certain brands of C. P. sodium chloride contain a toxic impurity whose destructive effect overbalances any beneficial effect the sodium chloride itself may have. It should be noted that the Kahlbaum salt, which Williams and Jacobs found to be the least toxic of the five brands tested, was used in these experiments.

indicated in Table I. The first, Chalkley solution,[3] contained nine chemical elements in the form of salts, the rest contained fewer. Then 20 cc. of the solution under consideration was put into each of five 50 cc. pyrex glass beakers. Numerous amœbæ were now removed from a stock culture and put into a pyrex glass beaker containing 25 cc. redistilled water and left a few minutes, after which all that were in good condition were transferred to another pyrex glass beaker containing redistilled water. This was repeated a third time. Then 10 ± amœbæ, washed free of culture fluid, were put into each beaker containing the different salt solutions. Specimens of *Chilomonas* were now concentrated by means of a centrifuge; they were then added to a large quantity of redistilled water and again concentrated, after which 0.2 cc. of the resulting dense culture of *Chilomonas* was added to each beaker. This organism served as food for the amœbæ.

During the process of washing in redistilled water, practically all of

TABLE I

Chemical Composition of the Solutions Tested as Culture Media for Amœba proteus

Compound	(1)	(2)	(3)	(4)	(5)
	gram	*gram*	*gram*	*gram*	*gram*
NaCl	0.08	0.08	0.08		
$NaHCO_3$	0.004	0.004	0.004		
KCl	0.004	0.004		0.004	0.004
$CaCl_2$	0.004	0.004	0.004	0.004	0.004
$CaH_4(PO_4)_2$	0.002	0.002	0.002	0.002	0.002
$Mg_3(PO_4)_2$	0.002		0.002	0.002	0.002
$Ca_3(PO_4)_2$					0.002
H_2O (cc.)	1000	1000	1000	1000	1000

the original culture fluid with its unknown chemical content was eliminated from both amœbæ and *Chilomonas*. No nutrient in the form of hay or grain was added to the salt solutions in the beakers. Therefore, since the composition of *Chilomonas* was the only unknown chemical factor in these solutions and since this factor was the same in all, any variation in the vital processes in *Amœba* must be due to known differences in the chemical constitution of the solutions.

In both series of experiments, all of the beakers were kept in diffuse light. Each was covered with a glass plate to reduce evaporation. The temperature during the course of the tests was fairly constant and was the same for all. Observations with reference to the number of amœbæ and their physiological condition were made with a binocular microscope. The condition of *Chilomonas* was also noted. These

[3] This solution is a modification by Chalkley of one used by Drew in tissue culture work; it has proven a reliable culture medium for *Amœba proteus*.

observations were made every day or every other day during the first half of the experiments; a final observation was made several days later, at which time the experiments were discontinued. The amœbæ became quite numerous in some of the solutions and the process of counting them became increasingly difficult. The latter was facilitated by marking quadrants on the bottom of the beakers with a China marking pencil and then counting the amœbæ in each section separately.

In the first experiment three salt solutions were tested as follows: (1) Chalkley solution, (2) Chalkley solution without $Mg_3(PO_4)_2$ (Table I), and a mixture of half Sieur de Mons spring water with half redistilled water.[4] The experiment continued 21 days, but at the end

TABLE II

Effect of Different Salt Solutions on Growth in Amœba proteus

Solution	Days after inoculation					Average progeny per original individual
	0	4	10	17	21	
	Number of animals present					
(1) Chalkley solution	41	72	214	446	696	16.9
(2) Same less $Mg_3(PO_4)_2$	42	72	179	249	263	6.26
Mixture of half-spring-half-redistilled water	45	75	147	267	310	6.8

of a week, specimens of *Chilomonas* were not abundant in the solutions; consequently, 0.2 cc. of a fresh, washed, and concentrated culture was added to each beaker.

The hydrogen-ion concentration of all the cultures remained between pH 6.3 and 6.6 during the experiment. The results obtained regarding the number of amœbæ present are given in Table II. Each number in the first five columns of this table is the total number of amœbæ in the five beakers of the stated solution at the stated time. Each number in the last column is the average progeny produced from each original amœba by successive fissions during the course of the experiment; it is obtained by dividing the number in column five by the corresponding number in column one.

This table shows that there was growth in each of the three solutions, as indicated by increase in the number of individuals, and that

[4] Ordinary spring water diluted with distilled water is usually a favorable medium for culturing amœbæ. In this experiment water from the Sieur de Mons spring, Mt. Desert Island, was used as the control solution. This spring water is a very weak solution of various salts ordinarily found in soil. Chalkley solution, however, was found to be a more favorable medium.

Amœba proteus can live and reproduce for more than 21 days in a synthetic salt solution with *Chilomonas paramecium* the only organic material present. It indicates that Chalkley solution is the most favorable, and Chalkley solution without $Mg_3(PO_4)_2$ is the least favorable of the three tested. The results obtained with spring water are of interest only in comparison and need not be considered further.

In the second experiment four solutions were tested in the same way as those in the preceding experiment. These salt solutions are described in Table I; *i.e.*, (1) Chalkley solution, (3) Chalkley solution without potassium salt, (4) Chalkley solution without sodium salts, and (5) Chalkley solution with the sodium salts replaced by calcium tribasic phosphate. The hydrogen-ion concentration of (1) and (3) remained between pH 6.4 and 6.6 and that of (4) and (5) between pH 6.2 and 6.4 during the 17 days of the experiment. The omission of sodium bicarbonate in (4) and (5) is responsible for this difference in hydrogen-ion concentration.

TABLE III

Effect of Different Salt Solutions on Growth in Amœba proteus

Solution	Days after inoculation					Average progeny per original individual
	0	2	4	6	17	
	Number of animals present					
(1) Chalkley solution	50	97	225	563	1956	39.1
(3) Same less potassium salt	50	85	210	565	1686	33.7
(4) Same less sodium salts	50	80	210	577	2404	48.1
(5) Same with sodium salts replaced by $Ca_3(PO_4)_2$	50	94	233	656	2676	53.5

The results obtained regarding the number of amœbæ produced are presented in Table III. The numbers have the same significance as in the preceding table. By referring to this table, it will be seen that there was little variation in the number of amœbæ present in the different solutions during the first part of the experiment but that there was considerable variation during the last part.

This variation on casual observation may not appear great, but when subjected to statistical analysis, it shows certain relations of some significance. Since the data were obtained on cultures (*i.e.*, groups of ten amœbæ) instead of on individuals, the mean progeny in five cultures of a solution was chosen as the relative value for that solution. The means with their probable errors and the difference between the means with their probable errors, computed from the same data as Table III, are given in Table IV.

It is considered reasonably certain that the mean of several measurements falls within three times its probable error; *i.e.*, the chances are 22.5 to 1 that it does. Table IV shows that the means of cultures (1) and (3) and also those of (4) and (5) are separated by about twice the sum of their probable errors whereas those of (1) and (4) are separated by more than four times the sum of their probable errors. Computation of the difference between the means shows the same relations; the difference between the means of (1) and (3) is 2.73 times the probable error, that between (4) and (5) 1.73 times, and that between (1) and (4) 5.25 times. It will be noted also that when the other solutions are compared with Chalkley solution, each of the differences between the means is probably significant, *i.e.*, in comparing (3) with (1), it is 2.73 times the probable error, (4) with (1) 5.25 times, and (5) with (1) 4.19 times. This indicates that the difference between (1) and (3) and also between (4) and (5) may or may not be

TABLE IV

Means and difference between the means of number of amœbæ present in the different solutions. Solutions arranged in ascending order of means. Based on same data as Table III.

Solution	Mean amœbæ per culture with p.e.	Difference between means with p.e.	$\frac{M_1-M_2}{p.e.}$
(3) Chalkley less K-salt	337.2±15.55		
		54.0±19.76	2.73
(1) Chalkley	391.2±12.26		
		89.6±17.06	5.25
(4) Chalkley less Na-salts	480.8± 7.03		
		54.4±31.47	1.73
(5) Chalkley less Na-salts, plus Ca-salt	535.2±30.70	144.0±34.33	4.19

significant of an actual difference between the sample distribution in these solutions but that the differences between (1) and (4) and also between (1) and (5) are significant of actual differences and cannot be due to random sampling alone. Comparison of the distribution in the different solutions by the χ^2 method (*see* Pearson, 1914) indicates also that for any two solutions the variation in the number of amœbæ present cannot be due to random sampling alone. Consequently, since the cultures were set up with the same care, received the same treatment, and differed only in the salt content of the solutions, the observed variation in fission rate must be due, at least in part, to difference in the chemical composition of the solutions.

The data presented in Table III indicate therefore that Chalkley solution without sodium is more favorable than that with sodium, and

that Chalkley solution with the sodium salts replaced by calcium tribasic phosphate is still more favorable. It indicates also that the absence of potassium is detrimental because the amœbæ in the solution without it had the lowest fission rate of all. The difference in hydrogen-ion concentration of (4) and (5) as compared with (1) and (3) may be a factor in the more rapid fission rate in the former solutions, although the optimum is generally considered to be at pH 6.6–6.7.

In all solutions the amœbæ remained in good condition during the experiments; at each observation practically all were attached and moving in monopodal or bipodal form. Little change was observed in *Chilomonas* during the first week, and they were still in fair condition at the close of the experiments, although less rounded and plump than at the beginning. Detailed studies on the structural and physiological changes in *Chilomonas* during starvation are in progress.

Summary

1. *Amœba proteus* grows and reproduces for several weeks in a balanced salt solution containing potassium chloride, calcium chloride, calcium phosphate, magnesium tribasic phosphate, and *Chilomonas paramecium*.

2. They also grow in other solutions, but not so well; *e.g.*, in a solution containing sodium chloride and sodium bicarbonate in addition to the above salts.

3. The results obtained indicate that the presence of sodium is not only unnecessary but actually detrimental while that of magnesium and potassium is favorable if not essential for growth and reproduction in *Amœba proteus*.

4. By observing the effect on fission rate and other physiological processes of omitting various elements or altering their concentration in the solution, it is possible to ascertain the relative importance of the elements to the rhizopod protoplasm and the number and kind and amount necessary for growth.

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THE FORMATION AND STRUCTURE OF THE GLOCHIDIAL CYST[1]

LESLIE B. AREY

ANATOMICAL LABORATORY, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL

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INTRODUCTION

An important phase in the development of fresh-water mussels is the obligatory period of parasitism spent upon appropriate fish hosts. While superficially encysted on such hosts the tiny larval 'glochidium' transforms into a free-living juvenile mussel, more complex in internal structure but without any corresponding increase in external size. The purpose of the present communication is to record the events incident to the formation of the glochidial cyst, to describe the structure of the cyst throughout parasitism, and to examine the morphological relations existing between parasite and host to subserve metabolic functions. A preliminary report was published some years ago (Arey, 1923). Data for the hookless group of glochidia have been drawn chiefly from an intensive study of infections of *Lampsilis luteola* on the gills of the large-mouth black bass (*Micropterus salmoides*), and of *Lampsilis anodontoides* on the long-nosed gar (*Lepisosteus osseus*). Similarly, the hooked series comprised stages of *Hemilastena ambigua* on the gills of the urodele *Necturus*, and stages of *Anodonta corpulenta* on the fins of the orange-spotted sunfish (*Lepomis humilis*).

Closely graded stages of encystment are easily procured by intro-

[1] Contribution No. 168. Published by permission of the United States Commissioner of Fisheries. Acknowledgment is due the staff of the Fairport Biological Station for many helpful courtesies extended during the prosecution of this inquiry.

ducing the host into a small aquarium into which ripe glochidia have been placed. Attachment follows quickly, and samples of the gills or fins bearing glochidia can then be removed at intervals as desired. Such samples of *L. luteola*, *A. corpulenta* and *H. ambigua* were fixed promptly in Zenker's fluid. The *L. anodontoides* stages were preserved in Bouin. All the material was sectioned serially in paraffin at 6 μ and stained with hematoxylin and eosin.

Attachment of Glochidia

The tiny bivalved glochidium (0.3 mm. or less in size) is incapable of locomotion when liberated from the maternal gill. Chance alone brings it in contact with suitable hosts. If a fin or gill filament becomes inserted momentarily between the valves so that the chimney-like hair cells are touched, the glochidium snaps shut vigorously, pinching the intercepted tissue.[2] The more delicate, hookless group of glochidia attaches to the soft gill filaments, but the sturdy, hooked glochidia can pierce the fins as well. The process of attachment may be observed on excised gill filaments or fins placed with glochidia in a watch glass under the microscope.

Hookless Glochidia.—The sharp edges of the valves cut cleanly through the gill epithelium, affording surprisingly little evidence of hemorrhage or seeping from the incision. The location of the parasite on the gill filament governs the character of the bite. Those that attach to the blade-like edge of the filament cut through the epithelium and, usually, considerable connective-tissue stroma as well.[3] This is characteristic of most well-attached larvae, for these enclose a liberal amount of the deeper tissues. After the epithelium is passed the valves continue to close, cleaving and compressing the underlying stroma. The softer tissues are cut; the tougher gill substance, especially that containing blood vessels, is merely pinched. The walls of the blood vessels and other resistant constituents are constricted at the level of the compression, and expand like an hourglass on either side (Figs. 1 and 2).

It appears that the valve rim cuts both epithelium and soft stroma with ease, but when it encounters the tougher elements, the rim buckles inward until it lies flat against the interior of the valve proper.[4]

[2] Arey (1921). This publication contains a full discussion of the factors involved in closure.

[3] Attachment along the edges of the filament is most favorable for easy and successful encystment. Many glochidia embed deeply in the firm gill substance, sometimes even half below the surface.

[4] The mechanisms involved in the operation of both flange and hook are described in full in a separate contribution (Arey, 1924).

This serves the very practical purpose of furnishing a broad zone of contact, while at the same time the glochidium is prevented from cutting itself entirely free (Figs. 2 to 5).

Attachment to the gill lamellae is essentially similar, but as thin-walled, vascular laminae are encountered in this instance, the chief factor is compression rather than incision. In a typical case the pinched lamellae converge inward toward the approximated valvular rims.

Hooked Glochidia.—The events during the attachment of hooked glochidia are comparable to those already described for the hookless type. Gill infections are practically identical, but fin parasites may lie wholly within the epithelium. The hooks flex much as do the flanges in the other group, but their effect is more local.[4] They pierce the host tissue like tongs, and then are inturned; the tissue is thereby held firmly, while the spines which beset the outside of the hook lock it still further.

Encystment

Generalities.—The process of attachment is completed almost instantaneously. Both incision and compression are accomplished in less than a second. As a result, the ventral edges of the valves sink somewhat below the surface level of the host tissue (Fig. 1). There next ensues a period during which the glochidium is overgrown by the contiguous cellular tissue of the host. Successive stages of this are shown in Figs. 2 to 5. The covering-in process is rapid. In summer the black bass completes its gill cysts in about 3½ hours; yet I have observed fully formed cysts as soon as 2½ hours after attachment, and well advanced stages at one hour. Excised filaments in watch glasses may encyst glochidia even quicker than under normal conditions; two hours has been found sufficient to complete the process. The response in the gar-pike is slower than in the black bass, but encystment has been observed in three hours. Lower temperatures retard the reaction proportionately.

Glochidia which attach to gill lamellae do not form cysts as readily as those on the thicker edges of the filament. This is doubtless due to the amount of material available, as will be explained presently. On the same gill the lamellar cysts may demand twice the time taken by those along the filament's edge. Lamellae adjacent to the glochidium may unite by fusion to form the basal part of the cyst, which is then roofed over in the usual way.

At first, cysts tend to be somewhat thick, irregular and unsymmetrical (Fig. 5). Within two or three days they usually become thinner, smooth contoured and even (Fig. 6). My observations are in

complete agreement with Young (1911) on this point; it is strange that Schierholz (1889), Faussek (1901) and Harms (1907) have all described the cyst as originally thin and only gradually gaining thickness. When glochidia acquire a weak attachment and clasp but a small shred of host tissue, encystment is commonly unsuccessful and the glochidium is lost.

The Method of Cyst Formation.—It is natural to assume that direct proliferation of the cells of the host tissue provides the material for the cyst that encloses the glochidium. Indeed, this assertion is presented as the correct explanation of encystment by several observers (Young, 1911; Lefevre and Curtis, 1912; and still earlier workers). That such an explanation is both inadequate and contrary to fact has been the topic of another publication by the present writer (Arey, 1932*a*). The *a priori* argument against encystment through cell multiplication rests on several facts: (1) The cyst may be composed of several thousand cells; (2) the time required for the formation of a cyst under favorable conditions is only three to four hours; (3) the mitotic cycle is relatively slow and consumes several hours in cold-blooded vertebrates.

Actual observation of encystment stages does not show the presence of more than the ordinary number of random mitoses seen in control, uninfected tissue. For example, in 78 cysts of *Lampsilis luteola* on the black bass, representing stages between 30 minutes and 9 hours after attachment, a total of only 20 positive mitotic figures and 14 doubtful ones were found as the result of a thorough census under the highest magnification. Again, in 17 cysts of *Hemilastena ambigua* on the gills of *Necturus* only one mitotic figure occurred during the period of encystment.[5] These results definitely disprove the theory of encystment through the proliferation of new cells.

Turning now to the real factor underlying encystment, the natural alternative method is actually encountered. This process is one of cell migration, whereby neighboring host cells assemble and actively push forward over the invader until the wound is closed and the glochidium is covered in.[6] After encystment is complete there may be a compensatory period of cell division in the vicinity of the cyst to replace the cells lost during the cellular emigration leading to cyst formation. For the details of this process, and its relation to wound healing in general, the reader is referred to the complete publication already mentioned (Arey, 1932*a*).

[5] My material did not include stages beyond cysts three-fourths completed.

[6] In gill infections on fishes the cyst wall is composed both of epithelium and connective tissue (Figs. 5 to 8). Goblet cells or pigment cells are frequently carried along into the cyst. (Figs. 5 to 8).

The Structure of Cysts

The cyst is repeatedly designated as 'epithelial' by authors who have written on these matters. This, however, expresses only a half truth. Fin parasites, to be sure, may lie entirely within the stratified epithelium, and the same is true for some of the *Hemilastena* cysts on *Necturus* gills. But even in these cases there is commonly attachment to fin rays or connective-tissue stroma which necessitates a more or less extensive defect in the epithelial covering at its base.

Parasites on the gills of fishes usually bite deep into the stroma. Not only does connective tissue adjoin the glochidium here but it is carried up into the roof of the cyst as well, so that the larva in reality lies embedded in stroma (Figs. 2 to 4).[7] Often the epithelium forms a mere external arching canopy. The demarcation between epithelium and cellular connective tissue is commonly very indistinct, and the latter is easily mistaken for the former.[8] Doubtless this circumstance accounts for the existing confusion and erroneous statements concerning the composition of the cyst wall, for in some locations the interpretation is indeed puzzling and the two do appear to blend. Yet

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EXPLANATION OF PLATE

Abbreviations

a.m., adductor muscle of glochidium	*g.c.*, goblet cell
c.t., connective tissue of cyst	*g.f.*, gill filament
c.w., cyst wall	*gl.*, glochidium
ep., epithelium of cyst	*h.t.*, host tissue
f., flange of valve	*l.m.*, larval mantle

Fig. 1. Glochidium of *Lampsilis luteola* just attached to a gill filament of the black bass. Photo. × 150.

Fig. 2. An early stage in the encystment of *L. luteola* (30 minutes after attachment). Photo. × 300.

Fig. 3. A half-formed cyst enclosing *L. luteola* (1¼ hours after attachment). Photo. × 300.

Fig. 4. A cyst nearly completed about *L. luteola* (2¼ hours after attachment). Photo. × 300.

Fig. 5. The complete encystment of *L. luteola* (3½ hours after attachment). Photo. × 300.

Fig. 6. The wall of a *L. luteola* cyst at five days. Photo. × 355.

Fig. 7. The wall of a *L. luteola* cyst at four days. Photo. × 700.

Fig. 8. Tangential section of the wall of a large *L. luteola* cyst on a black bass with acquired immunity (21 hours after attachment). Photo. × 370.

Fig. 9. Gill filament of the black bass. Notches indicate the former location of sloughed *L. luteola* glochidia. Photo. × 19.

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[7] Deep-lying melanophores have been found in the cyst wall. In some cysts, especially those associated with immunity, eosinophils also invade the stroma (Arey, 1932*b*).

[8] Mallory's connective tissue stain does not differentiate these tissues in fishes.

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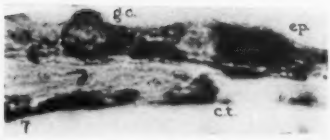
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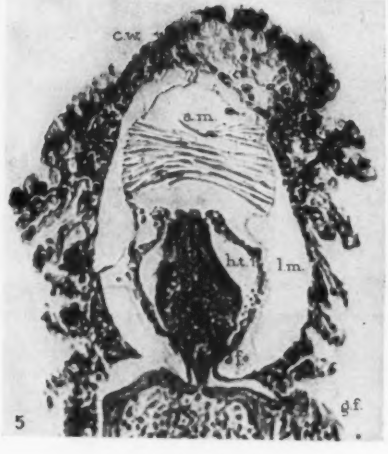
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in favorable preparations the demarcation is clear (Figs. 6 to 8); in the *Lampsilis anodontoides* series on the gar this differentiation was especially evident. Corroborative proof lies in the fact that delicate blood vessels may course through the cellular stroma, and in immune animals eosinophils wander freely through it (Arey, 1932*b*).

Correlative to these findings, the propriety of the term 'cyst' may be questioned as an exact designation for all glochidial investments. If by 'cyst' is meant a distinct envelope which demarks the larva from the adjacent tissues, then such does not exist. In the fin parasites the glochidium simply lies buried in the epithelium, or partly in the connective-tissue stroma. In the gill parasites the position is primarily in the stroma, with a roof-like canopy of epithelium outside. Yet the term is so thoroughly established and convenient as to make its replacement unwise.

The original irregularities of the cyst (Fig. 5) smooth over, and after a few days the roof tends to appear stretched and compact (Figs. 6, 7 and 9). Usually the distinction between epithelium and connective tissue then becomes plainer (Figs. 6 to 8). The goblet and pigment cells carried up in gill infections persist there (Figs. 6 and 7).

Relation of the Glochidium to its Host

Since the glochidium cannot metamorphose except on appropriate hosts it might be thought that special nutritive relations are established between parasite and host, and that this results in recognizable morphological changes or adaptations in the enveloping tissue. As a matter of fact, this possibility is not realized (Figs. 6 to 8). The soft, and for the most part highly vascular, tissues in which the parasite is embedded are apparently adequate for handling whatever interchanges are necessary without any special elaborations. The host tissue ingested at the time of attachment, together with the degenerating larval adductor muscle, are important sources of nutriment during transformation (Arey, 1932*c*), so that there is no metabolic 'strain.' The adjoining host tissues do not become unusually vascularized (Figs. 7 and 8) except in the *Proptera* glochidial type which is peculiar in that it undergoes marked increase in size during a postmetamorphic period of retention. In some specimens of *Proptera laevissima* which had increased in bulk some 40 times, the cysts were found to be very large and thick, and capillaries were present that presumably represented secondary invasive growths.

The Rupture and Repair of Cysts

When the cyst first forms, its wall is regionally variable in thickness and usually bears irregular outgrowths (Fig. 5). After a day or two it

becomes smooth and quite symmetrical. The tissue over the top gradually assumes a compact and stretched appearance, and the wall as a whole is thinner (Fig. 6). This reduction and thinning is much more spectacular in the bulky cysts which characterize the brief attachment of glochidia on immune hosts or non-hosts. In a contribution (Arey, 1932*b*) specifically describing these conditions it will be shown that the thinning is apparently due to the removal of cells back into the filament, rather than to a loss by desquamation or otherwise.

After the first days of encystment there are no especially significant changes in the cyst until the time when the glochidia are shed. Young (1911) has described a characteristic loosening of the cyst tissue and a concomitant infiltration of lymph after about one week of parasitism. A mild degree of cellular separation, in which intercellular bridges become prominent, occurs also in some of my series. Nevertheless, this is by no means a regular phenomenon, while sometimes it is observed relatively distant from an encysted glochidium as well. To what extent such alterations are artefacts and what their proper interpretation may be must remain unanswered at present. Miss Young suggested a causal relation to the premature sloughing of partly transformed glochidia. This may be true, but if so it is not a characteristic method by which these parasites terminate a normal period of encystment.

At the end of the parasitic period the glochidium becomes free of the host.[9] It has not increased in external size, but internally the metamorphosis is marked. Liberation is partly the result of the young mussel's own activity, for at intervals prior to detachment the valves may be observed to move and the foot to be pushed about, pressing the cyst wall. This is demonstrable when at this time filaments are removed and kept in watch glasses under a microscope; incidentally, there is reason to suspect that emergence is accelerated by such *in vitro* procedure. The cyst is eventually ruptured, but sections do not show that this is made perceptibly easier by any sudden terminal thinning or weakening of the wall. Portions of the old cyst-covering may be carried away and adhere for a time to the freed glochidium. Apparently a certain amount of gross sloughing aids the shedding process, for when infections are made on immune fish, transformation of the glochidium fails and the passive glochidium is liberated while still encysted (Arey, 1932*b*).

The freeing of the transformed glochidium leaves a defect in the filament which is rapidly filled in (Fig. 9), probably by the same sort of

[9] Those at the tips of gill filaments are often retained longest (Fig. 9). This is conceivably due to their less favorable position for receiving nutrition or oxygen.

cell mobilization that characterized encystment. Sections are not particularly informative on this point, and even the sites of the cysts are not easily detectable in microscopic preparations. In some instances the cavity of the remnant of the former cyst is temporarily filled with a coagulable exudate.

Summary

Mechanical stimulation of the larval glochidium induces non-selective, automatic closure upon the impinging gill or fin. The valves largely cleave the soft tissues encountered, but merely clasp such tough elements as blood vessels and fin rays which lie deeper. As a result, part of the glochidium is buried in host substance.

The glochidium is then covered by host tissue which advances from all sides, primarily for the purpose of closing the wound. Encystment is not the result of cell proliferation. On the contrary, it is accomplished by a mass movement of cells from the adjoining regions, advancing by their own activities and directed over the exposed valves by thigmotaxis. A compensatory period of mitosis may appear subsequent to encystment, apparently to replace cells lost to the cyst by emigration.

Fin cysts are largely epithelial in structure. Glochidia which attach to gill filaments lie embedded in cellular connective tissue, roofed over with an epithelial canopy.

Shortly after encystment is completed the cyst becomes thinner, smoother and more symmetrical. Thereafter, and even until the time of rupture, there are no further significant morphological changes in the cyst. Special adaptations of the host tissues to care for the wants of the metamorphosing parasite are not developed.

The glochidium is liberated partly through its own efforts, apparently aided somewhat by sloughing. Repair of the resulting defect in the host tissue is rapid and probably follows the general method utilized at encystment. This would involve an early redistribution of existing cellular elements, followed later by the formation of new cells to restore the tissue balance.

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